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(54) Title: PROTON GATED ION CHANNEL PROTEINS			
(57) Abstract			
Recombinant, isolated, enriched or cell free protein having activity as a proton activated receptor when expressed in, inter alia, mammalian dorsal root ganglion cells; to DNA and RNA encoding for expression of such protein in cells; cells containing this DNA and/or RNA and which express the protein in a form that is activatable by protons and its agonist analogues whereby the proteins exhibits electrophysiological and pharmacological properties characteristic of ASIC β and ASIC γ proton-gated cation channels; and DNA and RNA designed to downgrade expression of this protein for the purpose of gene therapy of pH mediated pain or disorders.			

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PROTON GATED ION CHANNEL PROTEINS

The present invention relates to recombinant, isolated, enriched and/or cell free protein having activity as an acid sensitive ion channel (ASIC) in, inter alia, mammalian cells; to recombinant DNA and RNA encoding for production of such protein in cells; cells containing such DNA and/or RNA which when expressed produces the protein in a form that is activated by presence of extracellular acid pH to allow passage of cations whereby the protein exhibits electrophysiological and pharmacological properties characteristic of a proton gated acid sensitive cation channel (ASIC); and DNA and RNA designed to downgrade expression of this protein for the purpose of gene therapy of acid pH mediated pain or disorders.

Tissue acidosis is a naturally occurring phenomenon that occurs in ischaemic, damaged or inflamed tissue. The reduction in pH in response to such events can be dramatic. In patients with intermittent claudication, the intramuscular pH can drop to 6.0 during exercise, whilst in an experimental model of cardiac infarction the pH of the cardiac circulation was reduced to pH 5.7 (Jacobus W.E., Taylor G.J., Hollis D.P., Nunnally R.L. (1977) *Nature* 265, 756-758). Associated with these conditions is a feeling of pain. This can be reproduced experimentally by infusion of low-pH solutions into skin (Steen, K.H., and Reeh, P.W. (1993). *Neurosci. Lett.* 154, 113-116.; Steen, K.H., Reeh, P.W., Anton, F., and Handwerker, H.O. (1992) *J. Neurosci.* 12, 86-95.) or muscle (4. Steen, K.H., Steen, A.E., Kreysel, H.-W., and Reeh, P.W. (1996). *Pain* 66, 163-170).

The pain caused by acids is thought to be mediated in part by H⁺-gated cation channels present in sensory neurons. A H⁺-gated channel (ASIC, for acid-sensing ionic channel) that belongs to the amiloride-sensitive Na⁺ channel/degenerin family of ion channels has recently been cloned (Waldmann *et al.* *Nature* (1997) 386, 6621, p173-177). Heterologous expression of this ASIC induces an amiloride-sensitive cation (Na⁺ > Ca²⁺ > K⁺) channel which is transiently activated by rapid extracellular acidification. The biophysical and pharmacological properties of the ASIC channel closely match one of the H⁺-gated cation channels described in sensory neurons but ASIC is also distributed widely throughout the brain.

In vitro studies have shown that low extracellular pH can evoke inward currents in both central nervous system and peripheral sensory neurons. Krishtal O.A., Pidoplichko V.I. (1980)

Neuroscience 5(12):2325-2327 demonstrated that low-pH evoked inward currents in rat trigeminal ganglion neurons, and similar observations have been made in rat dorsal root ganglion (DRG) neurons (Kovalchuk Yu.N., Krishtal O.A., Nowycky M.C. (1990) Neurosci Lett 31; 237-242 ;Bevan S., Yeats J. (1991) J. Physiol (Lond) 433:145-161; Zeilhofer H.U., Kress M., Swandulla D. (1997) J Physiol (Lond) 503(Pt 1):67-78). Low-pH responses from DRG are characteristically multi-phasic in nature suggesting the existence of distinct types of channel (e.g. Bevan and Yeats *ibid*). The molecular cloning of a number of proton-gated channels supports this conclusion.

Three mammalian proton-gated channels have been cloned recently; ASIC, (acid-sensing ionic channel) (Waldmann et al., *ibid*), DRASIC (dorsal root ASIC) (Waldmann R, Bassilana F, de Weille J, Champigny G, Heurteaux C, Lazdunski M. (1997) J Biol. Chem. 272(34):20975-20978), and MDEG-1 (a mammalian degenerin homologue) (Garcia-Anoveros J, Derfler B, Neville-Golden J, Hyman B.T., Corey D.P. (1997) Proc Natl Acad Sci U S A 94(4):1459-1464; Lingueglia E, de Weille JR, Bassilana F, Heurteaux C, Sakai H, Waldmann R, Lazdunski M (1997) J Biol Chem 272(47):29778-29783). A modulatory subunit, MDEG-2 has also been found (see Lingueglia et al.). All of these channels belong to the degenerin/ENaC channel superfamily, and are composed of two hydrophobic segments, intracellular N and C terminals, and a large extracellular loop (Garcia-Anoveros J, Corey DP (1997) The molecules of mechanosensation. Annu Rev Neurosci 20:567-594). ASIC, DRASIC, and MDEG1 each form functional channels when expressed in COS-7 cells. Evidence for heteromultimer formation between ASIC and MDEG has also been obtained (Bassilana, F., Champigny, G., Waldmann, R., de Weille, J.R., Heurteaux, C., and Lazdunski, M. (1997). J.Biol. Chem. 272, 28819-28822). The electrophysiological properties of these channels are diverse, as is their cellular localization. ASIC and MDEG-1 are widely expressed in nervous tissue, whilst DRASIC is found not only in sensory neurons, but also in the brain and spinal cord. The precise role of proton-gated channels in the central nervous system is as yet unknown.

The present inventors have now cloned ASIC DNA from a rat sensory neuron cDNA library and isolated and characterised three ASIC clones. In doing this they have determined the existence of two new splice variants of ASIC, $-\beta$ and $-\gamma$, having subtly different electrophysiological properties when heterologously expressed in COS cells. At least one of these is expressed only in sensory neurons, ie. peripheral sensory neurons.

Because of the specificity of expression and the importance of proton-gated channels in pain and inflammation, these new channel variants are very attractive analgesic drug targets and have utility, *inter alia*, in screening techniques for identifying novel analgesic and anti-inflammatory agents. Thus the present inventors have now provided the molecular cloning and characterisation of proteins that are new members of the ASIC ion channel family, herein designated ASIC proteins. *In situ* DNA hybridisation and RNA Northern blotting show that at least one of these new channel transcripts is present in dorsal root ganglion neurons and absent in other tissues. When expressed in transfected COS cells, the two channels mediate a flow of current when exposed to extracellular acidity, e.g. of about pH 5.

The alternative splicing of ASIC results not only in different gene products, eg. ASIC- α and ASIC- β , but also in different ASIC transcripts each of which has a distinct 5' UTR. The coding region of both ASIC- α transcripts corresponds to that of ASIC. The 5' heterogeneity of ASIC- α and ASIC- β may be generated initially by transcription from different promoters which may be tissue specific. In addition, we found two other splice variants of ASIC. One has a 29 amino acid deletion between codons 74 and 102 of ASIC- α . The other has a 600 bp insertion between codons 236 and 237 of ASIC- α . This insert causes a premature stop in translation and results in a new ASIC-like protein that has a shorter and unique C-terminal. We named this channel ASIC- γ and have shown that it forms a functional proton-gated channel when expressed in COS cells (data not shown). However, the level of expression of the ASIC- γ transcript is low, and we were not able to detect it on Northern blots.

As well as ASIC splice variants, two other proton-gated channels DRASIC and MDEG2 are also found in sensory neurons. Such heterogeneous expression of proton-gated channels may imply a complex response of sensory neuron sub-populations to tissue acidosis. All ASIC splice variants and DRASIC alone can form functional channels when expressed in COS cells, so that they may be able to function individually or combine with others to form heteromeric channels. Although the heteromultimerisation of these proton-gated channels has not been demonstrated, a recently isolated modulatory subunit MDEG2 has been shown to form heteromultimers with DRASIC which result in altered channel properties. It is possible that this kind of modulatory subunit may interact with other proton-gated channels in sensory neurons.

At present, ASIC- β is the only proton-gated channel which has been shown to be

exclusively expressed in sensory neurons. DRASIC and ASIC- α also show high levels of expression in DRG, but are also found outside the spinal ganglia. The DRG-specific expression of ASIC- β suggests that this transcript may arise from alternative splicing of a pre-mRNA generated by a DRG-specific promoter. Similar alternative splicing is also found in MDEG1 and MDEG2, which have the same splicing site as ASIC- α and ASIC- β , but neither MDEG1

nor MDEG2 is sensory neuron-specific. It will thus be interesting to analyse the ASIC- β promoter and compare it with other DRG-specific promoters. The specific splicing of ASIC- β mainly occurs in large diameter neurons which are different from the neurons expressing ASIC- α in DRG. The unique expression pattern of ASIC- β is also different from that of other known DRG-specific genes.

The expression of ASIC in brain and DRG has previously been examined using non-isotopic *in situ* hybridisation with a probe containing an L1 repeat. Using the L1-containing probe to screen a DRG random-primed cDNA library, we found about 2,500 positives from a pool of 200,000 clones, compared to 9 positive clones when using an ASIC-specific probe under identical conditions. This suggests that the L1 repetitive sequence exists in both ASIC and many other transcripts. We therefore used unique N-terminal coding regions to construct specific probes to investigate the expression of different ASIC splice variants in sensory neurons. Our *in situ* hybridisation studies reveal that 90% of ASIC- α positive cells are small diameter peripherin positive neurons, most of which are nociceptors. The results also show that ASIC- β is expressed in about 20% of the total number of neurons, and is found in both small and large diameter neurons.

The functional properties of the proton-gated channels so far described can be grouped broadly into two categories. Firstly, there are those channels which show a rapid time course for activation and inactivation in response to low pH, and secondly there are those which activate and inactivate much more slowly. ASIC- α is typical of the former group, whilst DRASIC falls into the latter. We found that the kinetic properties of ASIC- β are very similar to those of ASIC- α . The currents were quick to reach a maximum, and desensitised in the continued presence of low pH. The pH-dependency of currents passing through the two channels was also similar; EC₅₀ for ASIC- α was ~pH 6, versus pH 5.9 for ASIC- β . Both channels also show a preference for Na⁺. In addition, reversal potential studies showed that the ASIC- β channel was also permeable to K⁺ ions, although much less so than to Na⁺.

The major difference between the electrophysiological properties of ASIC- α and ASIC- β is related to the calcium permeability of the respective channels. ASIC- α discriminates poorly between cations, and the channel will pass Ca^{2+} ions, although the channel is 2.5 times more permeable to Na^{+} . However, low-pH evoked currents passing through the ASIC- α channel are inhibited at high extracellular calcium concentrations $>100\mu\text{M}$. In our study, we found that the ASIC- β channel was not permeable to calcium ions, nor did raising the extracellular calcium concentration become inhibitory. ASIC- β mediated currents thus exhibit similar properties to the native fast pH-evoked current recorded from voltage-clamped DRG neurons in response to low pH. It seems possible that ASIC- β mediates currents which contribute to the fast proton-activated current in sensory neurons. DRASIC-mediated currents are slow and sustained, similar to the sustained pH-mediated currents recorded from DRG neurons. However, we found that in addition to DRG, transcripts for DRASIC were also present, albeit at lower levels, in superior cervical ganglia, spinal cord, and brain stem, where sustained proton-evoked currents have not been described. ASIC- β is thus the only identified proton-gated channel expressed exclusively in sensory neurons.

Amiloride inhibited ASIC- β mediated currents in the COS cells with a similar efficiency to that seen with ASIC- α mediated current. This may indicate that the binding site for amiloride in the respective channels is in a conserved region. This region is presumably not present in the DRASIC channel, since amiloride produces potentiation of currents passing through this channel. It has been suggested that the selective neurotoxin capsaicin and protons may activate the same channel in sensory neurons. The recent molecular cloning of the capsaicin-gated channel VR1, suggests that this is not the case, since VR1 mediated currents were reported not to be activated by low pH. We found that capsaicin was not able to activate ASIC- β when expressed in the COS cells. These findings, taken together with other studies of cloned proton-gated channels, suggest that proton-gated and capsaicin-gated channels are different molecular entities.

Almost all (80-100%) DRG neurons have been shown to respond to low pH stimulation, but only half of them appear to be nociceptors. It thus seems likely that in addition to sensing tissue acidosis, proton-gated channels have other roles. ASIC- β is the first cloned proton-gated channel to be found in a subset of large diameter sensory neurons, in addition to the smaller, putative nociceptors. Drawing on models derived from studies of C. elegans mechanosensitive

mutants, it is possible that ASIC- β could be a component of mechanosensitive channels that need to be tethered to the cytoskeleton in order to function. Proton-gated channel transcripts are also present throughout the central nervous system, and it is difficult to identify a role for these channels in this region simply in terms of acid sensing. It is possible that these channels play a role as autoreceptors, given the acidic content of synaptic vesicles. It is also possible that the channels may be activated by other endogenous ligands or by mechanosensory stimuli. Heteromultimerisation of proton-gated channel subunits may produce channels with novel properties. It is also possible that proton-gated channel subunits may combine with ligand-gated ion channels (e.g. P2X receptors which also have 2-transmembrane domains) yielding a completely different repertoire of channel and receptor properties.

Thus in a first aspect of the present invention there is provided recombinant and/or isolated and/or enriched and/or cell free protein having the electrophysiological and pharmacological characteristics of an acid sensitive ion channel (ASIC) when expressed by eucaryotic cells, particularly when located in cell membranes, characterised in that the protein comprises an amino acid sequence having at least 80% sequence identity with that of SEQ ID No 2 or SEQ ID No 4 given in the sequence listing provided herein. Preferably the amino acid sequence differs from SEQ ID No 2 or SEQ ID No 4 only by conservative substitutions. More preferably the protein comprises an amino acid sequence having 90% or more, still more preferably 95%, sequence identity with SEQ ID No 2 or SEQ ID No 4 and optimally 100% identity with those sequences.

It should be noted that SEQ ID No 2 and SEQ ID No 4 are that of ASIC designated herein ASIC- β and ASIC- γ respectively; these having sequence homology of 78.7% and 74% with ASIC- α , the ASIC of Waldmann *et al*, shown as SEQ ID No 5 herein, as calculated using the GCG Wisconsin Software package incorporating FASTA and BLASTn software.

By the term identity is meant that the stated percentage of the claimed amino acid sequence or base sequence is to be found in the reference sequence in the same relative positions when the sequences are optimally aligned, notwithstanding the fact that the sequences may have deletions or additions in certain positions requiring introduction of gaps to allow alignment of the highest percentage of amino acids or bases.

Algorithms and software suitable for use in aligning sequences for comparison and

calculation of sequence identity will be known to those skilled in the art. Significant examples of such tools are the Pearson and Lipman search based FAST and BLAST programs. Details of these may be found in Altschul et al (1990), J. Mol. Biol. 215: 403-10; Lipman D J and Pearson W R (1985) Science 227, p1435-41. Publically available details of BLAST may be found on the internet at '<http://www.ncbi.nlm.nih.gov/BLAST/blast-help.html>'.

In a second aspect of the present invention there is provided recombinant and/or isolated and/or enriched and/or cell-free DNA or RNA encoding for the expression of a protein of the invention. Preferably the DNA or RNA is cDNA or cRNA and more preferably is characterised in that where it is a DNA it is a polynucleotide comprising nucleotide sequence having at least 80% identity with SEQ ID No 1 or SEQ ID No 3, as listed in the sequence listing herein, or a sequence having degenerate substitution of codon nucleotides in that sequence, and where it is an RNA it has a complementary sequence wherein T is replaced by U. Preferably the identity is 90% or more, more preferably 95% or more and most preferably 100%. Preferably non-identical parts of the sequences comprise degenerate substitutions.

Most preferred DNA or RNA is that which is capable of hybridizing with at least one poly- or oligonucleotide of sequence selected from sequences of SEQ ID No 1 and SEQ ID No 3 and polynucleotide and oligonucleotide fragments thereof of 15 or more contiguous bases, preferably 30 or more, selected from a characteristic region of these sequences with respect to ASIC- α , under high stringency conditions, more preferably being capable of such hybridization with two or more of these polynucleotides or oligonucleotides. Most suitable selections of sequences for performing these hybridizations will be selected from the unconserved coding regions of SEQ ID No 1 and 3 with respect to ASIC- α encoding sequences.

Thus with reference to Figures 3 and 7 below it will be seen that only amino acids 87 to 91 and 120 to 144 are fully conserved between ASIC- α and ASIC- β and thus these sequences as such are unsuitable for use in selecting hybridizing sequences for detecting ASIC- β homologs. In the case of ASIC- γ the most characteristic nucleotide sequences will be found encoding for amino acids 247 to 320, thus any probe incorporating a sequence selected from this region may be so used.

The expression 'high stringency conditions' will be understood by those skilled in the art, but are conveniently exemplified as set out in US 5202257, Col 9-Col 10, which is incorporated herein by reference.

The expression 'degenerative substitution' refers to substitutions of nucleotides by ~~those which result in codons encoding for the same amino acid; such degenerative~~ substitutions being advantageous where the cell or vector expressing the protein is of such different type to the DNA source organism cell that it has different codon preferences for transcription/translation to that of the cDNA source cell. Such degenerative substitutions will thus be host specific.

The expression 'conservative substitutions' as used with respect to amino acids relates to the substitution of a given amino acid by an amino acid having physicochemical characteristics in the same class. Thus where an amino acid in the SEQ ID No 2 or SEQ ID No 4 has a hydrophobic characterising group, a conservative substitution replaces it by another amino acid also having a hydrophobic characterising group; other such classes are those where the characterising group is hydrophilic, cationic, anionic or contains a thiol or thioether. Such substitutions are well known to those of ordinary skill in the art, i.e. see US 5380712 which is incorporated herein by reference, and are only contemplated where the resultant protein has activity as an ASIC protein.

For the purpose of producing proteins of the invention it will be possible to amplify template DNA from dorsal root ganglia cells using a specific DNA amplification reaction with specific primers targeted to amplify the DNA required, e.g. of SEQ ID No 1 or SEQ ID No 3, e.g. from genomic DNA, dorsal root ganglion cell DNA, cDNA or mRNA templates, e.g. by using polymerase chain reaction or, from RNA, by using reverse transcription (RT) followed by polymerase chain reaction (PCR). The RNA sequence can be derived from the DNA sequence and vice versa. Suitable primers for production of DNA of the invention will, for example, be of 10 to 30 nucleotide bases long and be complementary to the 5' and 3' ends respectively of the template, eg. target template sequence, e.g. of sequences SEQ ID No 1 or 3 as is conventional in the art. Use of reduced stringency binding conditions for the primer binding step of PCR allows, inter alia, amplification of less homologous DNA. For example, at 2 x SSC at 65°C the minimum interstrand complementary homology allowing hybridization can be calculated to be 76%.

Where it is desired to screen tissue derived libraries of genomic DNA or cDNA or mRNA for DNA or RNA of the invention it will be convenient to use low and/or high stringency hybridization techniques as indicated above. Such screening may be carried out using hybridization probes targeted at all or part of SEQ ID No 1 or 3. Alternatively ~~specific amplification primers (i.e. PCR primers) as described above may be used to~~ amplify 80% or more homologous sequences native to the source DNA to be used as PCR template and the product DNA may be used as a specific probe when labelled to identify DNA of the invention from the genomic, cDNA or mRNA library material. Such probes and primers are provided by a further aspect of the invention and may be used in Southern or Northern blotting procedures.

For the purpose of broad screening it may be preferred to use the probes, as described above, or as derived from PCR or RT/PCR of a sample DNA or RNA using primers as described above, under low stringency hybridizing conditions in order that a broad range of sequences might be identified having a good degree of homology with the respective part of the SEQ ID No 1. For the purpose of identifying DNAs with still higher homology, ie. over 80% identity with SEQ ID No 1 or 3, higher stringency conditions might be used or the probe used should comprise a larger part, e.g. substantial part and especially all, of the SEQ ID No 1 or 3.

For the purpose of identifying DNA coding for other ASIC proteins of the invention it will be preferred to use probes and primers capable of hybridizing with the highly conserved regions of the SEQ ID No 1 or 3.

For the purpose of precisely identifying DNA closely related to the proteins of the present invention it will be convenient to use probes or primers corresponding to, ie. identical to, all or a major part of the sequence of SEQ ID No 1 or 3, or to those parts of these sequence that are not well conserved between the different types of ASIC channel protein.

Suitable sequences for probes and primers of the invention will occur to those skilled in the art by alignment of the present sequence SEQ ID No 1 or SEQ ID No 3 with the other known ASIC protein sequence, the ASIC identified by Waldmann *et al.* It will be realised that such probes and primers may be targeted at either strand of this double stranded DNA in either case. Choice of probes and primers may be facilitated by reference

to factors such as those described in 'Lathe R: Synthetic Oligonucleotide Probes Deduced from Amino Acid Sequence Data: Theoretical and Practical Considerations: J. Mol. Biol (1985) 183, 1-12'. Expression of such identified DNAs in a bacterial or eucaryotic cell within a vector system therein will then provide the protein variants falling within the scope of the invention.

A further method for producing the proteins, DNA and RNA of the invention that does not correspond exactly in sequence to those of SEQ ID No 1 to SEQ ID No 4 is provided by use of mutagenesis, particularly chemically induced mutagenesis, and more particularly site directed mutagenesis (SDM), a technique which is well known to those skilled in the art and one for which a number of commercial kits enabling its convenient performance are now provided. For chemical mutagenesis, for example, a vectorised DNA of SEQ ID No 1 or SEQ ID No 3 is exposed to a mutagenic material such as hydroxylamine. In the case of SDM, a PCR reaction is carried out on that DNA using a mutagenic primer, whereby DNA is produced which encodes for a protein different in sequence to SEQ ID No 2 or 4 at a few predictable or predetermined sites respectively. Thus in this manner any protein DNA or RNA variant covered by the scope of the present invention is completely enabled merely by processing known nucleic acid material to give a desired altered product.

In order to still better enable production of DNA, RNA and protein of the invention the present inventors have, on behalf of the applicant, deposited two E. coli X-L1⁺ ampicillin microorganisms containing a λ -Zap-II derived Bluescript SK+ plasmid incorporating the SEQ ID No 1 and SEQ ID No 3 respectively at the National Collections of Industrial and Marine Bacteria Limited (NCIMB), 23 Manchar Drive, Aberdeen, Scotland, AB2 1RY under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms of 1977; these being deposited on 13 August 1997 under accession numbers NCIMB 40891 - (ASIC- β) and 40890 -(ASIC- γ). Specific DNA provided by the examples below or as derivable from the deposited plasmids of the invention may be thus used as PCR template or as a source from which clones may be derived; the latter being particularly the case for the deposited plasmid.

In a third aspect of the present invention there is provided antisense DNA to the DNA of the invention which has utility in so called gene therapy in so far as it may be used

to downregulate production of these newly determined ASIC proteins *in vivo*. Examples of use of antisense nucleic acids for therapy may be found for example in WO 92/15680 and WO 95/02051, the US equivalents of which being incorporated herein by reference.

A first method using the antisense-DNA of the invention uses oligonucleotides, typically being of 10 to 30 bases long, conveniently about 20 bases long, optionally in degradation protected form, e.g. by being thiolated, and which conveniently have been chemically synthesized to be directed to hybridize with a part of the 5' coding region of the ASIC mRNA. Annealing with the oligomeric DNA causes the mRNA to be degraded by activation of RNase H, or blocks the translation of the mRNA into protein. The small size of such oligomers facilitates their direct access into target cells which express the present ASIC proteins. The sequence of such oligomers should be selected as antisense sequences to sequences from regions of SEQ ID No 1 and 3 which are not conserved with respect to DNA encoding for SEQ ID No 5.

A second approach produces antisense RNA *in vivo* by inserting a tissue specific inducible or constitutively active promoter, enhancer or locus control region or element upstream of the coding region, or part of the coding region, of antisense DNA to all, or characteristic parts of SEQ ID No 1 or 3 in a construct which is then cloned into a vector. For use in mammals such a vector should be capable of infecting but not killing target cells. Convenient such vectors for use in targeting mammalian dorsal root ganglion cells are Herpes Simplex Virus (HSV) vaccinia or adenovirus derived vectors.

Where the antisense downregulating DNA or RNA is provided in dorsal root ganglia cells it potentially inhibits the pain response by actually decreasing the number of ASIC channels on the surface of sensory cells. Methods for producing antisense DNA to the DNA of SEQ ID No 1 or SEQ ID No 3 and their partial sequences will involve no undue burden to those skilled in the art, while incorporation of this DNA into mammalian cells might be readily accomplished using vectors, e.g. such as HSV, vaccinia or adenovirus (see Principles of Gene Manipulation (1994) 5th Edit. Old and Primrose 5th Edition, Blackwell Scientific Publications).

A fourth aspect of the present invention provides hybridization probes and primers for use in identifying a DNA and RNA, and ultimately, by interpretation of the DNA code, RNA and protein, of the invention. Such probes and primers comprise oligonucleotides

comprising one or more sequences targeted to hybridize with all or part of either of sequences SEQ ID No 1 and 3, i.e. at least 10 contiguous bases of characteristic parts thereof which respect to ASIC- α encoding sequences. Preferred and unsuitable regions for selection from are described above with respect to hybridization. Conveniently these are selected to hybridize with either strand of the double stranded target sequences. Primers will preferably be of 10 to 30 bases long while probes may conveniently be 20 to 50 bases long.

A fifth aspect of the present invention provides recombinant DNA constructs, particularly vectors, capable of expressing the ASIC DNA of the invention when incorporated into the DNA of a host cell, or expressing the antisense DNA or RNA of the invention. Suitable vectors for expression of mammalian DNA will occur to those skilled in the art, e.g. HSV, adenovirus or vaccinia vectors as described above, or pcDNA3 shuttle vectors, e.g. as included within the lambda express system (Stratagene), which are capable of expressing heterologous protein in both bacteria and in eucaryotic cells such as COS cells. Suitable bacterial vectors will include λ -Zap vectors such as the λ -Zap-II vector available from Stratagene Cloning Systems. Bacterial clones containing plasmids capable of gene expression can be obtained by excising pBluescript from the λ -Zap-II construct in the presence of a filamentous helper phage also available from Stratagene. Typical protocols are provided in the examples below, in Stratagene kit inserts, and in Old and Primrose (ref above). Many other vectors suitable for expression of the DNA of the invention in heterologous recombinant form will occur to those skilled in the art.

A sixth aspect of the present invention provides cells transformed by the DNA or RNA of the invention such as to be capable of expressing, or having expressed, an ASIC protein as described hereinabove; these cells having electrophysiological and pharmacological identity with native ASIC- β or ASIC- γ expressing cells, particularly with cells bearing ASIC protein as specifically found in mammalian dorsal root ganglia. Such cells are provided by transformation of a host cell, preferably a eucaryotic cell, e.g. a COS, CHO or 293 cell or an oocyte, preferably a Xenopus oocyte, but particularly COS cells, using DNA of the invention as incorporated by recombinant DNA techniques into a vector (see e.g. Old and Primrose, pp330 and pp352) or as directly incorporated into the cells' genomic DNA e.g. by electroporation or other such DNA integrating technique.

It is also possible to produce cells bearing the receptor protein of the invention by direct injection of mRNA, corresponding to the cDNA of the invention, into the cells wherein it becomes translated; such mRNA is also referred to as cRNA, particularly when used in this manner. In the case of Xenopus oocytes it is possible to achieve the presence of functioning ASIC protein on cell membranes within 3 days such that it is ready for electrophysiological and pharmacological study.

A seventh aspect of the present invention provides a method for producing a cell of the invention comprising transforming a host cell, particularly of the aforementioned types, with nucleic acid of the invention, preferably in vectorised form.

An eighth aspect of the present invention provides a method for identifying a substance as being an ASIC protein agonist, partial agonist or antagonist comprising exposing the surface of a cell of the sixth aspect of the invention to a solution of the substance such as to allow interaction between the substance and ASIC protein in the membrane and then measuring the electrophysiological response of the cell to this interaction. Typically the response may be measured by use of a microelectrode technique accompanied by such measurement strategies as voltage clamping of the cell whereby activation of ion channels may be identified by inward or outward current flow as detected using the microelectrodes. ^{22}Na , ^{86}Rb , ^{45}Ca radiolabeled cations or ^{14}C or ^3H guanidine may be used to assess such ion flux; a sodium, calcium or potassium ion sensitive dye (such as Fura-2, or indo) may be used to monitor ion passage through the receptor ion channel, or an electrical potential sensitive dye may be used to monitor potential changes, e.g. such as in depolarization.

Agonists and partial agonists may be identified by their relative efficacy as compared to extracellular acid pH or other known agonists in activating the receptor or, in the case of partial agonists and antagonists, by their ability to block the activation caused by a given acidity, for example pH 5, or other known agonists.

The present invention will now be described by way of illustration only by reference to the following non-limiting Figures, Sequence Listing and Examples. Further embodiments of the invention falling into the scope of the claims provided herewith will occur to those of ordinary skill in the art in the light of these.

FIGURES

Figure 1. The gene structure of ASIC splice variants. Three different transcripts are distinguished by their 5'UTRs (hatched), but share the same 3'UTR (white). The coding regions are black apart from the unique N-terminal of ASIC- β (striped).

Figure 2. The proposed molecular structures of ASIC- α and ASIC- β shows that both proteins have the same transmembrane domain structure, with intracellular N and C terminals. The lightly shaded region of ASIC- β shows its unique N-terminal region including the first transmembrane domain.

Figure 3. The N-terminal sequence alignment of 4 ASIC-related proteins. Block letters represent the cysteine residues which are conserved amongst the 4 proteins implying a similar secondary structure.

Figure 4. Northern blots of ASIC- β distribution. The Northern blots were probed with N-terminal unique sequences of ASIC- α , ASIC- β , and DRASIC. All three proton-gated channels are expressed in sensory neurons. ASIC- α is distributed in many neural tissues and cell lines. There are three different sizes of ASIC- α transcripts in PC12 cells which are 2.5, 3.2, and 4.0 kb, but only one major transcript of 3.2 kb in sensory neurons. ASIC- β is only expressed in DRG as a 3.2 kb transcript. DRASIC is predominantly in DRG with two sizes of transcripts, 2.0 and 2.5 kb, but is also expressed in SCG, spinal cord, and brain stem. The relative amount of RNA loading is indicated by cyclophilin probe.

Figure 5. Characteristics of the pH response in COS-7 cells expressing ASIC- β .

5a: Typical response to low pH in ASIC- β transfected COS cells. The cell was voltage-clamped at -60mV and low pH applied at the bar. Dotted line indicates zero current level.

5b. pH-response relationship obtained from experiments similar to that in a. Responses were normalised against the maximal response, and plotted against the pH. The half-point for activation of the current was pH 5.9.

5c. Time taken for the current to activate and inactivate plotted against pH.

5d. Recordings made during a change in command potential using a linear ramp protocol (duration of ramp 240ms). Current was recorded under control conditions, and during application of pH 4. The current reverses at approximately +25mV.

Figure 6. Calcium-dependency and pharmacology of ASIC- β mediated currents in COS cells.

6a. Responses obtained to pH 5.1 (at the closed circles) in the presence of increased extracellular calcium concentration. Recordings were made from the same cell at intervals of three minutes.

6b. (left panel), control response to pH 5.1, (right panel), responses to low pH in the absence of extracellular sodium and increased calcium concentration. Current flowing via ASIC- β is not inhibited by extracellular calcium, nor is the channel permeable to calcium. Dotted line indicates zero current level.

6c. Amiloride inhibits ASIC- β mediated current. The IC₅₀ derived from this plot was 21 μ M.

6d. Capsaicin does not activate ASIC- β . Recordings made from the same cell, holding potential was -60mV. Upper trace shows that application of capsaicin (500nM) at the bar failed to evoke an inward current. pH 4.1, at the bar 3 minutes later (lower trace), evoked a robust inward current. Traces have been separated for clarity, and the dotted line indicates zero current for each recording.

Figure 7. Aligned amino acid sequences of ASIC- α (known), ASIC- β (first ASIC protein of the present invention derived from dorsal root ganglia) and ASIC- γ (second ASIC protein of present invention that is weakly expressed).

Figure 8. Plots of current (pA) against time showing the effect of exposure of transfected COS cells expressing ASIC- β and ASIC- γ respectively to extracellular pH of about 5.

SEQUENCE LISTING

The listing provided herewith gives the DNA and amino acid sequences of the following examples of the invention.

SEQ ID No 1: cDNA corresponding to ASIC- β protein as derived from Rattus dorsal root

ganglia cells.

SEQ ID No 2: Amino acid sequence of ASIC- β protein from Rattus dorsal root ganglia cells.

SEQ ID No 3: cDNA corresponding to ASIC- γ protein as derived from Rattus dorsal root ganglia cells.

SEQ ID No 4: Amino acid sequence of ASIC- γ protein from Rattus dorsal root ganglia cells.

SEQ ID No 5: Amino acid sequence of ASIC- α protein from Rattus.

SEQ ID No 6: Primer for use with that of SEQ ID No 7 or 8 in PCR screening for ASIC- β DNA .

SEQ ID No 7: Primer for use with that of SEQ ID No 6 in PCR screening for ASIC- β DNA .

SEQ ID No 8: Primer for use with that of SEQ ID No 6 in PCR screening for ASIC- β DNA.

SEQ ID No 9: Primer for use with that of SEQ ID No 10 in PCR screening for ASIC- γ DNA.

SEQ ID No 10: Primer for use with that of SEQ ID No 9 in PCR screening for ASIC- γ DNA .

EXAMPLES

Example 1: Isolation of ASIC- β and ASIC- γ DNA of the invention.

The mouse BnaC2 sequence (U78179; PNAS V.94, 1458-1464, 1997) was used to design ASIC-primers 5' ACTGTACTCCGGAGCAGTACAAGG-3' and 5'-GAGTTCCAGCACTGTGAGGATGCT-3' 20ng cDNA was synthesized from polyA+ RNA isolated from DRG of new born rat and was PCR amplified using ASIC-7 and ASIC-8 (35 cycles 94°C 1' -55°C 1' - 72°C 1'). A 330bp-fragment that was PCR-amplified from

rat DRG single stranded DNA was used to screen a full-length rat DRG cDNA library.

The PCR-amplified DNA fragments were labeled with ^{32}P (Gibco Rad-prime kit) and used as probes to screen a DRG cDNA library. 200,000 clones from a size-fractionated (2-4 kb) oligo(dT)-primed cDNA library from neonatal rat DRG were screened by hybridization with the PCR probes (25 ng, specific activity 2×10^9 cpm/ μg) in 4 x SSC containing 0.5% SDS, 5 x Denhardt's solution, 100 $\mu\text{g}/\text{ml}$ boiled salmon-sperm DNA, 10 $\mu\text{g}/\text{ml}$ poly(U), and 10 $\mu\text{g}/\text{ml}$ poly (C) at 65°C for 4 hours. The DRG cDNA filters were given a final wash in 0.2xSSC, 0.5% SDS at 65°C . In total, 32 positive clones were picked and analyzed by sequencing.

Northern Blot analysis: Specific N-terminal sequences of different ASIC clones were chosen as templates to synthesized cRNA probes. ASIC (nucleotide positions 750-1068 corresponding to amino acids 74-179) was subcloned into pGEM-3Z using Eco RI and Pst I sites; ASIC- β (320-700 corresponding to amino acids 18-163) was subcloned into pGEM-11Zf by Apa I sites. For DRASIC, a 380 bp DNA fragment were amplified by PCR using primers;

5'-GTGCGCCACTACACGCTATGCCAAGGAGC-3'

5'-GGGGAACATGTGTTTCGATGCCCATTCAAC-3'

and subcloned into T-vector (Promega); for cyclophilin, a 300 bp DNA fragment were amplified by PCR using primers;

5' ACCCCACCGTGTCTTCGAC-3'

5' CATTTGCCATGGACAAGATG-3,

and subcloned into T-vector. Antisense labelled cRNA was synthesized from these templates using SP6 RNA polymerase and [^{32}P]-UTP. Such cRNAs were used to probe Northern blots with 20-50 μg total RNA in each lane. Hybridization was carried out in 50% formaldehyde, 5 x SSC containing 0.5% SDS, 5 x Denhardt's solution, 100 $\mu\text{g}/\text{ml}$ boiled salmon-sperm DNA, 10 $\mu\text{g}/\text{ml}$ poly(U) and 10 $\mu\text{g}/\text{ml}$ poly(C) at 68°C for 24 hours, with a final wash in 0.1 x SSC with 0.5% SDS at 75°C .

In situ hybridization. The same templates used for probing Northern blots were labeled with digoxigenin-UTP (Schaeren-Wimers N. And Gerfin-Moser A. (1993) Histochemistry **100**, 431-440). After *in situ* hybridisation, sections were double-labeled with neuronal sub-population markers. Monoclonal antibodies against peripherin or N-52 (Chemicon International Inc. and

Sigma) were used at 1:500 dilution in blocking solution (1x PBS containing 10% sheep serum, 0.5% Triton X-100). FITC-conjugated secondary antibodies (Boehringer Mannheim) were used at a 1:200 dilution in blocking solution. For IB4 staining, the IB4-FITC (4 µg/ml, Sigma) was diluted in 1:300 PBS containing 0.1 mM CaCl₂, MgCl₂, MnCl₂, and 0.2% Triton X-100

(Molliver D.C., Wright D.E., Leitner M.L., Parsadanian A.S., Doster K., Wen D., Yan Q., Snider W.D. (1977) *Neuron* 19(4):849-861).

ASIC- α , as described by Waldmann *et al.*, is expressed in cortex, cerebellum, hippocampus, pituitary, trigeminal mesencephalic nucleus (3kb and 3.8kb bands); in DRG (only 3kb band); in PC12 (3.8kb, 3kb and 2.4kb bands); in cell lines ND7/23, ND8 and N-tera-neuro cell-lines (3.8kb). ASIC- α is not expressed in SCG, enteric neurons, sciatic nerves and other non-neuronal tissue.

ASIC- β , an ASIC gene comprising DNA corresponding to that of the present invention, is expressed only in DRG as a single 3kb band. The ASIC- β expression level in DRG sensory neurons is 2 or 3-fold less than expression of ASIC- α which is expressed 10 to 20 fold less than the purinergic receptor protein gene P_{2X3}.

ASIC- γ is expressed at a very low level and was not detectable by Northern hybridization.

The ASIC- α transcript encodes the 526 amino acid protein named ASIC, but has distinct 5' and 3' UTRs from the previously reported sequence. There are two types of 5' UTR in the DRG ASIC- α clones. The major population of ASIC- α (90%) have a 5' UTR of up to 530 bp which is GC-rich (73%). A small percentage (10%) of ASIC- α clones have a short 5' UTR (about 100 bp) that corresponds to the sequence reported for ASIC 5' UTRs found in brain. All the ASIC-related clones in DRG have an identical 3' UTR that is different from the ASIC UTR reported in brain. An L1-like repetitive sequence reported in ASIC- α is not found in DRG ASIC- α or β transcripts.

The longest open reading frame of ASIC- β is 513 amino acids in length, sharing the same 341 amino acids with the C-terminal of ASIC- α . The N-terminal 172 amino acids of ASIC- β are unique, with highest homology to DRASIC (43.8%), 39.7% identity to both ASIC- α and MDEG1, and 22.6% identity to the FMRFamide-gated sodium channel (FaNaC). These

five proteins share three conserved cysteines within the N-terminal region of the extracellular loop. There are two additional cysteine residues in ASIC- β when compared to DRASIC, ASIC- α MDEG1, and FaNaC, suggesting that the secondary structure of ASIC- β might have some unique features. N-terminal splicing at a similar position is also found in the related MDEG2 transcript, which differs in 236 amino acids from MDEG1, but this N-terminal sequence has no homology to the N-terminal of ASIC- α . Further homology cloning with different probes derived from ASIC-related clones, showed that the major transcripts represented in our DRG library are ASIC- α ASIC- β and DRASIC, but not MDEG1.

ASIC- α was found in many neuronal tissues, including DRG, spinal cord, trigeminal ganglia and the trigeminal mesencephalic nucleus. The cell lines, PC12, ND7/23 and N-tera2 also expressed ASIC- α (Fig. 2). In contrast ASIC- β , seen as a 3.2 kb transcript, was found only in the DRG and not in other tissues or cell lines. DRASIC has been reported to be a sensory neuron specific proton-gated channel. However, in addition to the DRG, we found low level transcripts of DRASIC in superior cervical ganglia, spinal cord, and also the brain stem. These data suggest that ASIC- β is the only proton-gated channel which is exclusively expressed in sensory neurons.

We next examined the cell type distribution of different ASIC splice variants in DRG. We used peripherin to label small diameter sensory neurons, and IB4 to label the neurotrophin independent cells that also comprise a large proportion of nociceptors. We used an anti-neurofilament antibody N-52 to define the large diameter neurons that are mainly mechanoreceptors and proprioceptors. Using 5' coding region probes of ASIC- α and ASIC- β for *in situ* hybridisation, we found that both ASIC- α and ASIC- β are expressed in 20-25% of L4 DRG neurons. The ASIC- α positive neurons are mainly small diameter cells (>90%) which co-express peripherin but not IB4. In contrast, ASIC- β positive neurones are composed of both small diameter and large diameter neurones, of which 70% express neurofilaments and only 30% co-express peripherin. These data demonstrate that ASIC- β exhibits both a tissue and cell specific distribution of expression that is clearly different to that shown by ASIC- α .

Example 2: Preparation of cDNA from deposited E. coli of NCIMB deposited strains.

Bluescript plasmid DNA encoding for the protein of the invention was purified

from the E. coli DNA by solvent extraction, Magic minipreps or Caesium chloride centrifugation, and digested with endonucleases EcoRI and XhoI targeted at the 3' polylinker downstream of the cDNA and the site of the vector bacteriophage polymerase.

Examples 3 and 4: Protocols for isolation of mammalian, e.g. human, sensory neuron-specific proton-gated cation channels (ASIC) using sequence derived from the rat cDNA clone.

Example 3: Homology Cloning

Dorsal root ganglia are isolated from available human or other mammalian source tissue. RNA is extracted from the isolated tissue by extraction in guanidine or other chaotropic agents, followed by solvent extraction using phenol, phenol/chloroform and precipitation using isopropanol and ethanol.

The isolated RNA is Northern blotted with probes derived from the ASIC- β and - γ receptor clone DNA of SEQ ID No 1 and/or 3 to identify transcript size of candidate human clones. Messenger RNA from the RNA pool is extracted using oligo-dT cellulose or poly-U sepharose chromatography and cDNA is constructed from this RNA using reverse transcriptase (SuperScript) and DNA ligase. Double-stranded DNA is constructed and a directional cDNA library generated using lambda zap, lambda express, pcDNA, or other suitable vectors.

The 3' untranslated region of the ASIC- β or ASIC- γ is sub-cloned into a plasmid vector (Bluescript or similar) followed by cutting out the insert and isolating it on agarose gels. cRNA clones are labelled by *in vitro* transcription with SP6, T3 or T7 polymerases and ^{32}P or DIG labelled nucleotides. Alternatively the insert is radiolabelled by random prime or nick translation with ^{32}P or DIG-labelled nucleotides

The cDNA library is screened by moderate stringency hybridisation to 50-60°C 5 x SSC, using radiolabelled or other labelled DNA or cRNA probes derived from the 3' UTR of the DNA/RNA sequence. Alternatively, other regions of the protein may be used. Resulting clones are plaque purified and their insert sized examined. Cross hybridisation of the clones follows with isolation of individual distinct clones. Clones that contain inserts that correspond approximately to the size of human mRNAs determined by

Northern blotting are isolated. cRNA is generated from the selected clones and injected into Xenopus oocytes or COS (up to 50nls, 1mg/ml) and using twin electrode voltage clamp studies, the expression of functional proton-gated channels investigated.

Functionally active channel clones have their insert DNA sequenced and this DNA is recloned into a selectable shuttle vector (e.g. pcDNA neo- Invitrogen) to generate transiently or permanently transfected cell lines (COS cells, CHO cells, 293 cells etc.) expressing functional human ASIC protein clones. These cell lines may then be used with either ^{22}Na , ^{86}Rb , ^{45}Ca ion flux measurement, electrophysiology or Na-sensitive dye techniques to provide a high-throughput screen for channel agonist or antagonist candidate compounds.

Example 4: PCR cloning

Dorsal root ganglia are isolated from available human or other mammalian tissue material. RNA is extracted from the isolated tissue by extraction in guanidine or other chaotropic agents, followed by solvent extraction using phenol, phenol/chloroform and precipitation using isopropanol and ethanol.

Random primers and reverse transcriptase are used to generate cDNA from the extracted human RNA using either total or poly A+ RNA.

Degenerate PCR primers derived from relatively conserved regions of SEQ ID No 1 or 3 are used to amplify the cDNA using the polymerase chain reaction; e.g. using degenerate primer sequences SEQ ID No 6 to 10. The products of the PCR reaction are separated on agarose gels and examined with products of the approximate predicted size being extracted and cloned into a pGemT vector. The clones are sequenced and the sequences examined for similarity with that of SEQ ID No 1 or 3. Candidate PCR fractions are used to screen a human cDNA DRG library as described in Example 2.

Example 5: *In vitro* expression of proton gated ASIC channels.

Plasmids derived from NCIMB 40890 (ASIC- γ) or NCIMB 40891 (ASIC- β) are cut with restriction enzyme EcoRI. The 2kb insert of ASIC- β and 2.5kb insert of ASIC- γ , which comprise the protein encoding regions, are isolated from low melting agarose gel, and subcloned into Invitrogen pTracer-CMV, which expresses GFP and pRK7 vectors

respectively between EcoRI restriction sites. The orientation of these constructs is determined by sequencing.

Shuttle vectors eg. pTracer-CMV or pRK7 containing ASIC- β and - γ were purified from maxipreps. These vectors were used to express proton-gated channels by transfecting permanent cell line COS-7 cells. Cultured COS-7 cells from a 100 mm petri dish (80-90% confluent) were trypsinised and resuspended in 350 microlitres of ice cold HEBS buffer. 20-30 μ g of plasmids of interest were dissolved with 150 μ l of HEBS buffer, then mixed with the COS-7 cell suspension in an electroporation cuvette and kept on ice to cool for 5 minutes. Meanwhile, the electroporator (Invitrogen) was set up at 250 μ F, 0 Ohm and charged for 3 minutes at 330V, 25mA, and 25W. The cuvette was flicked to resuspend cells and electroporation effected.

After transfection by electroporation, COS-7 cells were seeded in low density in a 30mm petri dish and cultured with 2ml MEM/10% FCS at 37°C for 2-3 days. The transfected cells were washed with buffer (mM NaCl 146, KCl 5, CaCl₂ 2, MgCl₂ 1, Glucose 10, HEPES 10 at various pHs) and using the whole cell configuration of the patch-clamp technique, currents evoked by perfusing with low pH buffer (pH 5) were measured at a holding potential of -60mV, with a 20 second perfusion duration of pH 5 buffer. Compounds to be assessed as agonists, partial agonists or antagonists of the ASIC channels were bath applied and inward current used as measurement of the activation or block of channels encoded by the transfected vectors.

Example 6: Electrophysiology.

Whole-cell voltage-clamp recordings (Hammill O.P., Marty A., Neher E., Sakmann B. And Sigworth F.J. (1981) Pflugers Archiv 391 1108-1112) were made 2-3 days after transfection. Membrane currents were recorded using an Axopatch 200B amplifier. Currents were low-pass filtered at 5kHz (4-pole Bessel filter), and digitized using a Digidata 1200 interface. Acquisition and analysis of currents was achieved using pClamp6 software. Pipettes were pulled from borosilicate glass (Clark Electromedical, Reading, UK), and had DC resistances of approximately 3m Ω when filled with the pipette solution. All recordings were made at room temperature (18-22°C).

The extracellular recording solution was composed of (in mM); NaCl 146; KCl 5; Glucose 10;

MgCl₂ 1, CaCl₂ 0.01. For extracellular solutions with pH values of 7.4–6.5, 10mM HEPES was used as the buffer, whilst for solutions of pH 6.5–4.0, 10mM MES was used to provide optimal buffering capacity over the wide pH range (3.5 units) required. The normal pH of the extracellular solution was 7.4. In some ion substitution experiments, extracellular sodium chloride was replaced with an equal amount of choline chloride. The effect of extracellular calcium concentration on low pH-evoked currents was investigated by substitution of choline chloride with an equal concentration of calcium chloride, in the absence of extracellular sodium ions.

Low pH solutions were applied via a U-tube (Bormann J. (1992) in Practical Electrophysiological methods ed Kettenman H. And Grantyn R. pp 136–140 Wiley-Liss New York) placed close (<1mm) to the cell of interest. The use of the U-tube ensured that the cell was completely bathed in the test solution ensuring that no buffering by the bulk extracellular medium occurred. Low pH solutions were usually applied for 10–20s, with at least 2 minutes between applications. The intracellular solution contained (in mM); KCl 120, NaCl 8, MgCl₂ 3, HEPES (free acid) 40, and BAPTA (free acid) 10, at pH 7.35.

Transfected cells were identified by the presence of GFP. Application of low pH to ASIC- β expressing COS cells at a holding potential of -60mV evoked rapidly activating inward currents (Figure 5a). The threshold for activation of the current was around pH 6.5, and the current was maximal at approximately pH 4.0. Figure 5b shows the mean pH-response curve recorded from 6 ASIC- β transfected COS cells. The half-point for activation of the current in this series of experiments was pH 5.9. The inward currents evoked in response to pH 4.0 were variable in magnitude (range was 0.272 \pm 8.41nA), the mean response was 2.39 \pm 0.33nA in 27 cells). In 83% (34 of 41) of fluorescent cells, a response was observed to the application of pH 4.0–4.5, whilst in 17 of 17 untransfected COS-7 cells, application of pH 4.0–4.5 evoked no change in membrane current.

The pH-activated currents normally reached peak amplitude in around 1s in response to the lower pH solutions, and rapidly inactivated (or desensitised) in the continued presence of low pH. The time taken to peak was related to the applied pH, with the quickest activation times occurring in response to the lowest pH. The effect of pH on the time taken for inactivation of the current showed a similar pH-dependency, but was much more marked (Figure 5c).

Ionic Basis of ASIC- β mediated Inward Current: The reversal potential for the rapid phase of the low pH activated current was established by using either a linear ramp voltage-clamp protocol, or by sequentially stepping the command potential to a range of values whilst evoking the inward current (Figure 5d). The command potential was ramped over a period of 240ms between potentials of -80 and +60mV. The speed of the ramp allowed us to make accurate recordings despite the rapidly activating and inactivating nature of the response. The reversal potential was found to be 26.1 ± 2.3 mV ($n=8$). If the channel were only sodium permeable, the reversal potential would be expected to be approximately +73mV, given the composition of the intra- and extracellular solutions used. It thus seemed likely that the channel was also permeable to other cations, most notably K⁺, because of its presence in such high concentration. Ion substitution studies confirmed that the channel was also permeable to K⁺. Replacement of extracellular sodium with choline abolished inward currents, but on some occasions, small outward currents were seen, confirming that the channel was permeable to potassium ions (data not shown). These currents were small because most recordings were made at a holding potential of -60mV, close to the reversal potential of potassium ions with the solutions used. Previous studies have demonstrated that increasing the extracellular calcium concentration reduced the magnitude of proton-activated inward currents passing through the ASIC channel. This was not the case for ASIC- β . Figure 6a shows that in the presence of 146mM NaCl, increasing the extracellular calcium concentration had no effect on the magnitude of the inward currents evoked by the application of pH 4.0 buffer. In addition, ASIC- β was found not to be permeable to calcium ions. In the experiment shown in Figure 6b, a response to pH 4.0 was obtained under control conditions (left panel), and then extracellular NaCl was replaced by choline chloride, and the cell exposed to pH 4.0 buffer in the presence of a range of extracellular calcium concentrations. On removing extracellular sodium, no inward current was detected in response to low pH, and even when the calcium concentration was increased to 50mM no inward current was detected. Figures 6a and b are representative of experiments on four cells. Sodium ions were not a cofactor for calcium permeability. We recorded reversal potentials for ASIC- β mediated currents with 140mM Na⁺ in the external medium, with or without 20mM Ca⁺⁺. The mean reversal potentials were 25.2 ± 2.3 mV ($n=4$) in the absence and 24.8 ± 2.8 mV ($n=4$) in the presence of Ca⁺⁺, demonstrating that the channel is impermeant to calcium, even if Na⁺ ions are present.

Pharmacology of ASIC- β mediated Currents: We investigated the effect of amiloride, a known inhibitor of other proton gated channels, on ASIC- β mediated currents. Cells were voltage-clamped at -60mV and given a 20 second exposure to pH 4.5 solution, first in the absence and then the presence of increasing concentrations of amiloride. The threshold concentration for inhibition of low pH-evoked currents was between 1 and 10 μ M. Figure 6c shows an inhibition-response curve for amiloride constructed from experiments on 6 cells. The data points were fitted with a single Boltzman function, giving an IC₅₀ of 21 μ M.

Capsaicin is known to have an excitatory action on small diameter sensory neurons. Moreover, it has been suggested that capsaicin and protons activate a similar ion channel. To investigate whether capsaicin could activate the ASIC- β channel, we exposed ASIC- β transfected COS cells to 500nM capsaicin. In 4 of 4 cells, capsaicin evoked no change in membrane current, whereas a subsequent application of low pH to the same cells produced characteristically large inward currents (Figure 6d).

CLAIMS.

1. Recombinant and/or isolated and/or enriched and/or cell free protein having the electrophysiological and pharmacological characteristics of an acid sensitive ion channel (ASIC) when expressed by eucaryotic cells, characterised in that the protein comprises an amino acid sequence having at least 80% sequence identity with that of SEQ ID No 2 or SEQ ID No 4.
2. Protein as claimed in claim 1 wherein the amino acid sequence differs from SEQ ID No 2 or 4 only by conservative substitutions.
3. Protein as claimed in claim 1 or claim 2 comprising an amino acid sequence having at least 90% identity with SEQ ID No 2 or 4.
4. Protein as claimed in claim 3 comprising an amino acid sequence having at least 95% identity with SEQ ID No 2 or 4.
5. DNA or RNA encoding for the expression of the protein as claimed in any one of claims 1 to 4.
6. DNA or RNA as claimed in claim 5 characterised in that it is cDNA or cRNA.
7. cDNA or cRNA as claimed in claim 6 characterised in that it comprises a polynucleotide of nucleotide sequence having at least 80% identity with SEQ ID No 1 or 3 or a sequence having degenerative substitution of codon nucleotides in one of those sequences or a complementary sequence thereto.
8. cDNA or cRNA as claimed in claim 6 comprising a polynucleotide sequence having at least 80% identity with SEQ ID No 1 or 3 or a sequence having degenerative substitution of codon nucleotides in that sequence.

9. cDNA or cRNA as claimed in any one of claims 6 to 8 wherein the identity of the sequence is of at least 90% to SEQ ID No 1 or SEQ ID No 3.

10. cDNA or cRNA as claimed in claim 9 wherein the identity is at least 95%.

11. cDNA as cRNA as claimed in any one of claims 5 to 10 characterised in that it is in recombinant or enriched or isolated and/or cell free form.

12. DNA or RNA as claimed in any one of claims 5 to 11 characterised in that the non-identical parts of the sequences comprise degenerate changes, deletions or additions.

13. DNA or RNA as claimed in any one of claims 5 to 12 characterized in that it is capable of hybridizing with at least one poly- or oligonucleotide of sequence selected from sequences of SEQ ID No 1 or 3 under high stringency conditions.

14. A method of producing a cDNA or cRNA encoding for a protein as claimed in any one of claims 1 to 4 comprising hybridizing PCR primers capable of hybridizing with the respective 5' ends of its two strands with mammalian genomic DNA or cDNA template DNA and extending the primers using polymerase chain reaction.

15. A method of producing a DNA encoding for a protein as claimed in any one of claims 1 to 4 comprising converting mammalian dorsal root ganglion mRNA to cDNA using reverse transcription (RT) and then amplifying the DNA encoding therefor using a the method as claimed in claim 16 with the RNA/DNA double stranded nucleic acid product as template.

16. A method of identifying a DNA or RNA as claimed in any one of claims 5 to 12 comprising screening a mammalian tissue derived library of genomic DNA or cDNA or mRNA for the occurrence of hybridization with hybridization probes targeted at all or part of SEQ ID No 1 or 3.

17. A method of identifying a DNA or RNA as claimed in any one of claims 5 to 16 comprising specifically amplifying the desired sequence using primers targeted at the 5' ends of the two strands of SEQ ID No 1 or SEQ ID No 3 using a mammalian tissue derived library of genomic DNA, cDNA or mRNA as PCR or RT/PCR templates and using the product DNA as a hybridization probe in a method as claimed in claim 16.

18. A hybridization probe or PCR primer comprising an oligonucleotide or polynucleotide of sequence capable of hybridizing with a polynucleotide of SEQ ID No 1 or 3 under low stringency conditions characterized in that it comprises 10 or more contiguous bases of a DNA or RNA as claimed in any one of claims 5 to 12 selected from a region not found in ASIC- α cDNA or cRNA.

19. A hybridization probe or primer as claimed in claim 18 characterised in that it is capable of hybridizing with a polynucleotide of SEQ ID No 1 or 3 under high stringency conditions.

20. A hybridization probe or primer as claimed in claim 17 or 18 characterized in that it is capable of hybridizing with one or more of the regions of the SEQ ID No 1 or SEQ ID No 3 that is not highly conserved with respect to ASIC- α .

21. A method for producing a protein as claimed in any one of claims 1 to 4 comprising expressing a DNA of any one of claims 5 to 12 in a bacterial or eucaryotic cell.

22. A method as claimed in claim 21 wherein the DNA is a DNA of SEQ ID No 1 or SEQ ID No 3 that has been altered by chemical or site directed mutagenesis to produce a different DNA, that also being a DNA as claimed in any one of claims 5 to 13.

23. A vector comprising a DNA or RNA as claimed in any one of claims 5 to 13.

24. A vector as claimed in claim 22 characterized in that it is a plasmid.

25. A vector as claimed in claim 23 characterized in that it is a λ -Zap-II derived bluescript plasmid..

26. A vector as claimed in claim 23 characterized in that it incorporates a recombinant DNA or RNA as claimed in any one of claims 5 to 13 as included in the microorganism deposits made on 13 August 1997 at the National Collections of Industrial and Marine Bacteria Limited (NCIMB) 23 Manchar Drive, Aberdeen, Scotland AB2 1RY under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms of 1977 under accession numbers NCIMB 40891 or NCIMB 40890.

27. A vector as claimed in claim 23 characterized in that it is capable of expressing a protein of any one of claims 1 to 4 when incorporated into a host cell.

28. A vector as claimed in claim 27 characterized in that the cell is a eucaryotic cell.

29. A vector as claimed in claim 28 characterized in that it is a vaccinia, adenovirus or herpes simplex virus vector or is a pcDNA3 shuttle vector.

30. Antisense DNA or RNA to cDNA or RNA as claimed in any one of claims 5 to 13.

31. Antisense DNA or RNA as claimed in claim 30 characterized in that it consists of an oligonucleotide of 10 to 30 bases long.

32. A vector capable of transforming eucaryotic cells characterized in that it incorporates a DNA or RNA as claimed in claim 31.

33. A vector as claimed in claim 32 characterized in that it is a vaccinia, adenovirus or herpes simplex virus vector.

34. A cell transformed by the DNA or RNA as claimed in any one of claim 5 to 12 or a vector of any one of claims 28 to 33, the cell being capable of expressing, or having

already expressed, an ASIC protein as claimed in any one of claims 1 to 4.

35. A cell as claimed in claim 34 having electrophysiological and pharmacological identity with ASIC protein bearing cells.

36. A cell as claimed in claim 35 wherein it has identity with cells bearing ASIC protein as specifically characteristic of mammalian dorsal root ganglia cells.

37. A cell as claimed in any one of claims 33 to 36 characterized in that it is a eucaryotic cell.

38. A cell as claimed in claim 37 characterized in that it is a COS, CHO or HEK 293 cell or an oocyte.

39. A cell as claimed in any one of claims 33 to 38 characterized in that it has mRNA, corresponding to the cDNA of any one of claims 5 to 13, incorporated within it.

40. A method for producing a cell as claimed in any one of claims 33 to 38 characterised in that it comprises transforming a host cell with a DNA, RNA or vector as claimed in any one of claims 5 to 13 and 22 to 28.

41. A method for identifying a substance as being a proton-gated ion channel agonist, partial agonist or antagonist comprising exposing the surface of a cell as claimed in any one of claims 33 to 39 to a solution of the substance such as to allow interaction between the substance and ASIC protein in the membrane and then measuring the response of the cell to this interaction and/or the response of the cell to presence of extracellular acid pH.

42. A method as claimed in claim 4 wherein the substance is a partial agonist or antagonists and is identified by its ability to block the response of the cell to presence in the solution of a given acid pH or any agonist.

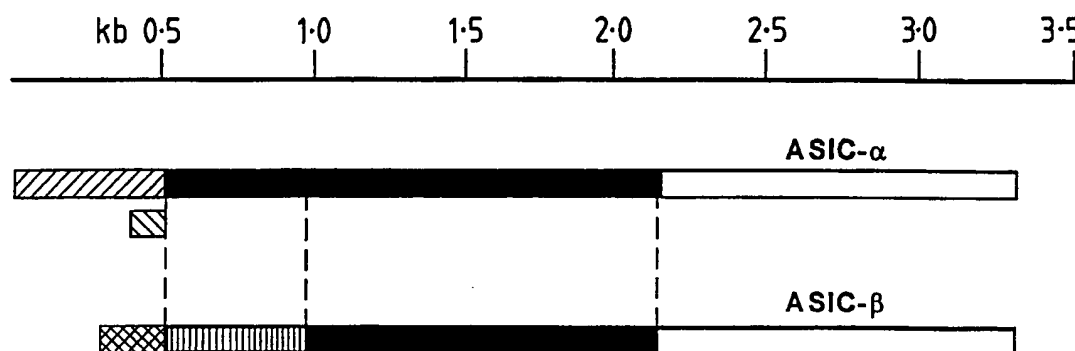


Fig.1

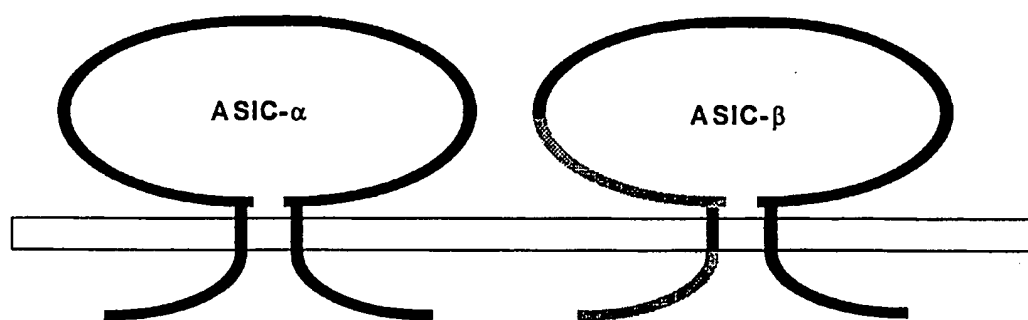


Fig.2

ASIC-alpha	MELKTEEEV	GGVQPVSIQA	FASSSTLHGL	AHIFSVERLS	LKRALWALCF	50
ASIC-beta	--AGS-LD-G	DDS.-RDLV-	--N-----A	S-V-VEGGPG	PRQ-----	49
DRASIC	-KPRSDL--A	QRR-ASD-RV	----C-M---	G---GPGG-T	-R-G---T-V	50
MDEG1	-D--ESPS-	-SL--S---I	--NT-----I	RHIFVYGP-T	IR-V---VA-	49
TM1						
ASIC-alpha	LGSLAVLLCV	CTERVQYYFC	YHHVTKLDEV	AASQLTFPAV	TLCNLNEFRF	100
ASIC-beta	VIA-GAF--Q	VGD--A--LS	-P---L-----	-T-E-V-----	-F--T-AV-I	99
DRASIC	-L---AF-YQ	VA---R--GE	F--K-T---R	ESH-----F--	----I-PL-R	100
MDEG1	V---GL--VE	SS---S---S	-Q-----	V-QS-V-----	-----G---	99
ASIC-alpha	SQVSKNDLYH	AGELLALLNN	RYEIPDTQMA	DEKQLEILQD	KANFRSEKPK	150
ASIC-beta	--L-YP--LY	LA.PMLG-D.ESDDP.	...GVPLAPP	GPEA...-SGE	137
DRASIC	-RLTP---HW	--TALLG-D.-AEHA-	YLRA-GQPPA	PPGF..MPSE	143
MDEG1	-RLTT-----	-----DV	NLQ---PHL-	-PTV--A-RQ	----KHY---	149
ASIC-alpha	PFNMRIFYDR	AGHDIRDMLL	SCHFRGEACS	AEDFK	185	
ASIC-beta	---LHR--N-	SC-RLE----	Y-SYC-GP-G	PHN-S	172	
DRASIC	T-D-AQL-A-	---SLE-----	D-RY--QP-G	P-N-T	178	
MDEG1	Q-S-L--LH-	V---LK--M-	Y-K-K-QE-G	HQ--T	184	

Fig.3

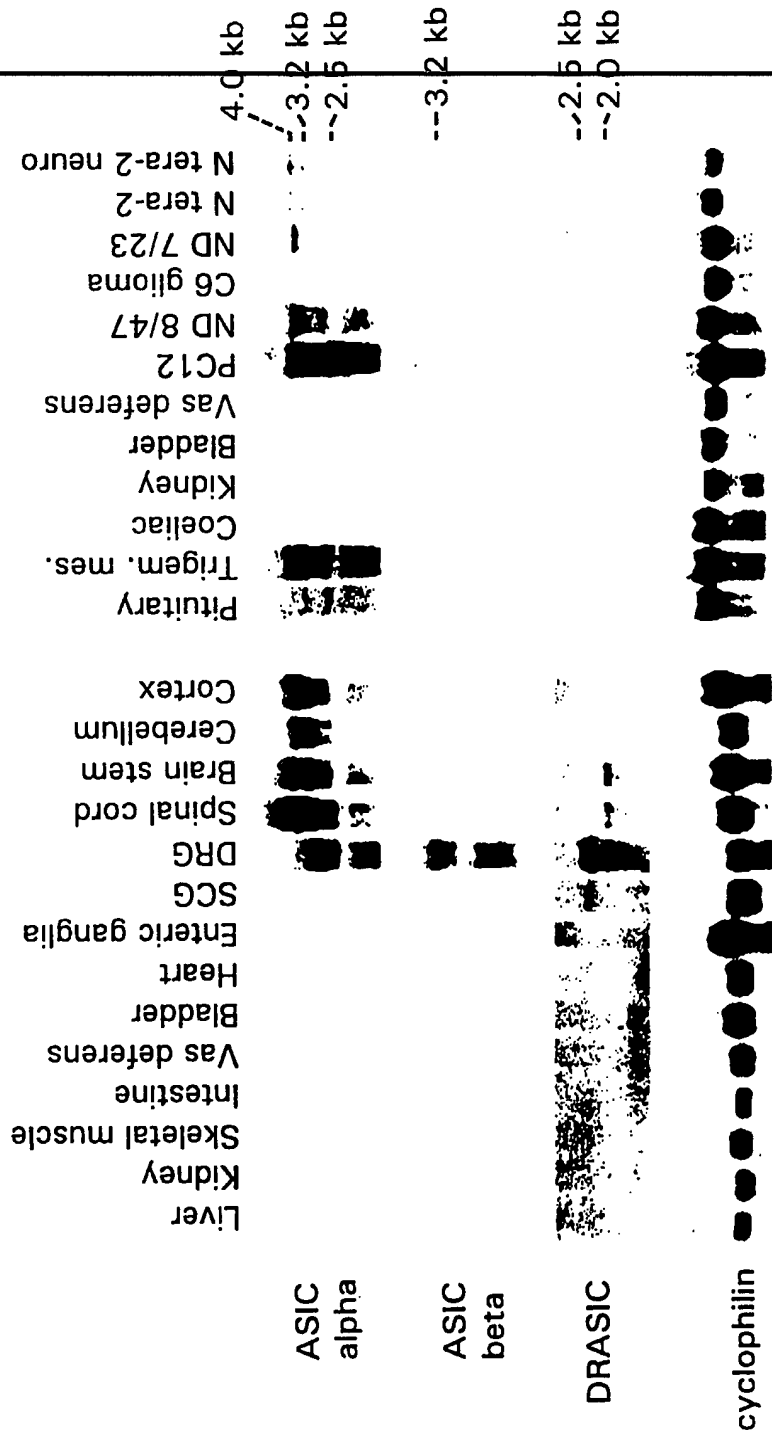


Fig.4

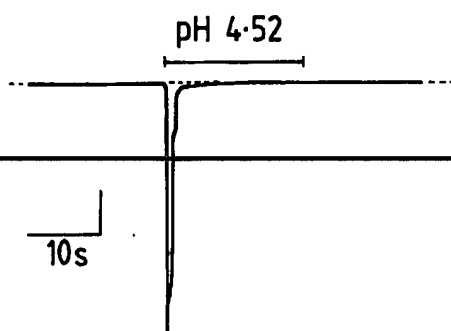


Fig. 5a

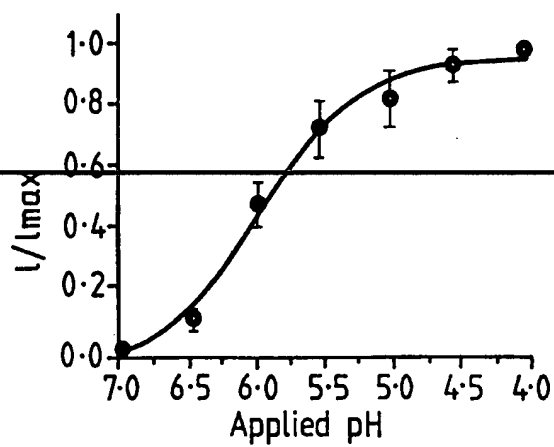


Fig. 5b

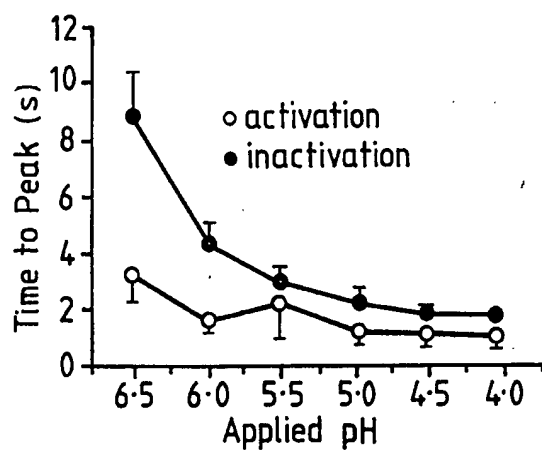


Fig. 5c

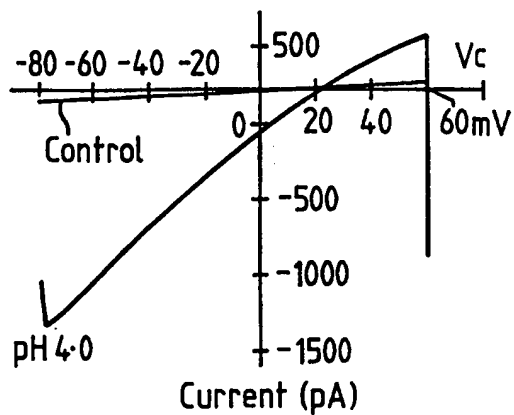


Fig. 5d

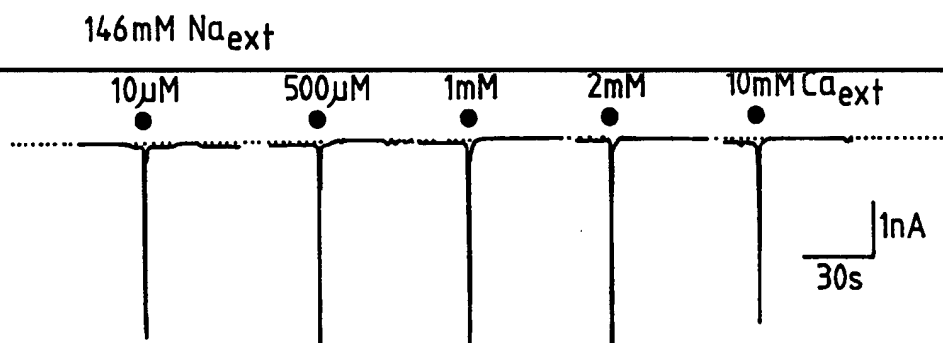


Fig. 6a

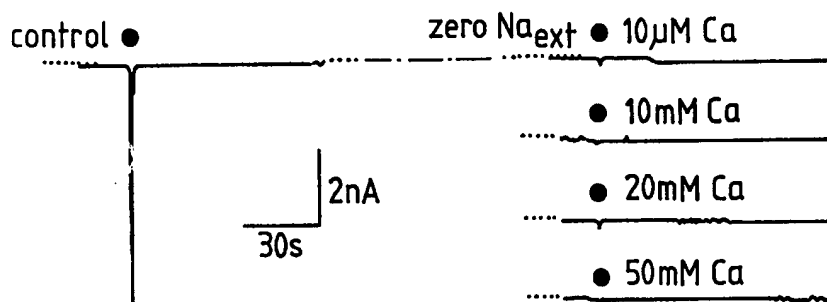


Fig. 6b

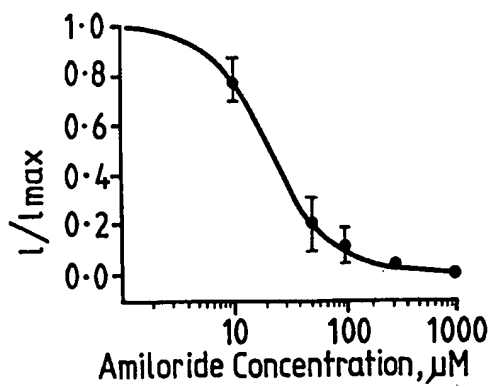


Fig. 6c

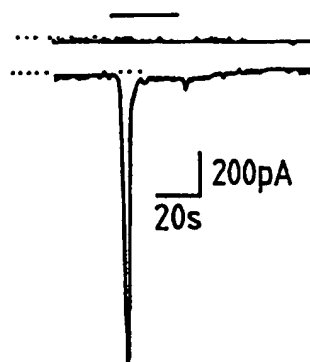


Fig. 6d

α M E L K T E E E E V G G V Q P V S I Q 20
 β - - A G S - L D - - D D S - R D L V
 γ - - - - - - - - - - - - - - - -

A F A S S S T L H G L A H I F S Y E R L 40
 - - - N - C - - - - A S - V - V E G G P
 - - - - - - - - - - - - - - - -

S L K R A L W A L C F L G S L A V L L C 60
 G P R Q - - - - V A - V I A - G A F - -
 - - - - - - - - - - - - - - - -

V C T E R V Q Y Y F C Y H H V T K L D E 80
 Q V G D - - A - - L S - P - - - L - - -
 - - - - - - - - - - - - - - - -

V A A S Q L T F P A V T L C N L N E F R 100
 - - T - E - V - - - - - F - - T - A V -
 - - - - - - - - - - - - - - - -

F S Q V S K N D L Y H A G E L L A L L N 120
 L - - L - Y P - - L Y L A P M - G - D E
 - - - - - - - - - - - - - - - -

N R Y E I P D T Q M A D E K Q L E I L Q 140
 S D D P G V P L A P P G P E A F S G E P
 - - - - - - - - - - - - - - - -

D K A N F R S F K P K P F N M R E F Y D 160
 F N L H R - - N
 - - - - - - - - - - - - - - - -

R A G H D I R D M L L S C H F R G E A C 180
 - S C - R L E - - - - Y - S Y C - G P -
 - - - - - - - - - - - - - - - -

Fig 7(i)

S A E D F K V V F T R Y G K C Y T F N S 200
G P H N - S - - - - - - - - - -
- - - - - - - - - - - - - - - -

G Q D G R P R L K T M K G G T G N G L E 220
- - - - - - - - - - - - - - - -
- - - - - - - - - - - - - - - -

I M L D I Q Q D E Y L P V W G E T D E T 240
- - - - - - - - - - - - - - - -
- - - - - - - - - - - - - - G M S

S F E A G I K V Q I H S Q D E P P F I D 260
- - - - - - - - - - - - - - - -
G L L K E H L P W A R L A P N S I R A P

Q L G F G V A P G F Q T F V S C Q E Q R 280
- - - - - - - - - - - - - - - -
L C S P G T K F R H V P F L P R S H H V

L I Y L P S P W G T C N A V T M D S D F 300
- - - - - - - - - - - - - - - -
C L C P Y V T S A L S V P W L L L P G L

F D S Y S I T A C R I D C E T R Y L V E 320
- - - - - - - - - - - - - - - -
V T L Q G N Q A S S S I S G R H S L Y K*320

N C N C R M V H M P G D A P Y C T P E Q 340
- - - - - - - - - - - - - - - -

Y K E C A D P A L D F L V E K D Q E Y C 360
- - - - - - - - - - - - - - - -

V C E M P C N L T R Y G K E L S M V K I 380
- - - - - - - - - - - - - - - -

Fig.7(ii)

P S K A S A K Y L A K K F N K S E Q Y I 400

- - - - -

~~G E N I L V L D I F F E V L N Y E T I E 420~~

~~- - - - -~~

Q K K A Y E I A G L L G D I G G Q M G L 440

- - - - -

F I G A S I L T V L E L F D Y A Y E V I 460

- - - - -

K H R L C R R G K C Q K E A K R S S A D 480

- - - - -

K G V A L S L D D V K R H N P C E S L R 500

- - - - -

G H P A G M T Y A A N I L P H H P A R G 520

- - - - -

T F E D F T C * 526

- - - - - * 513

Fig.7(iii)

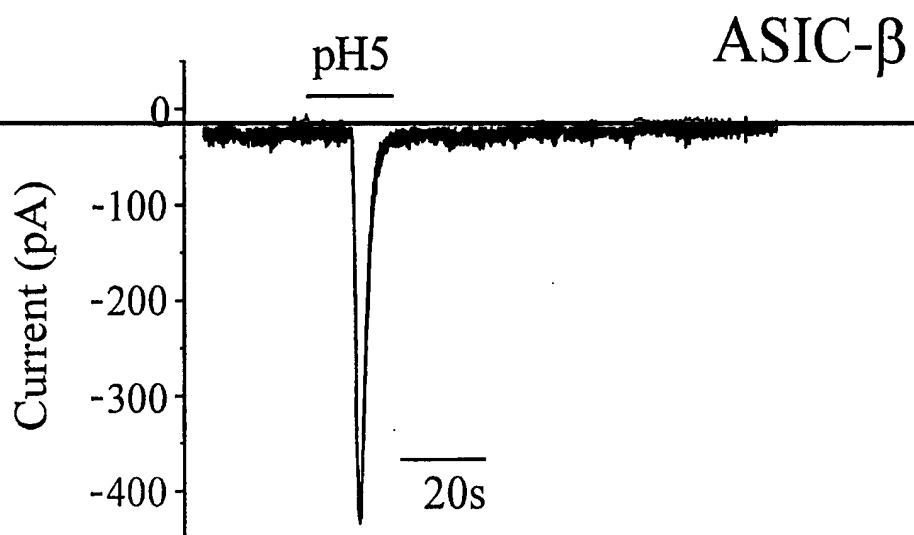


Fig.8a

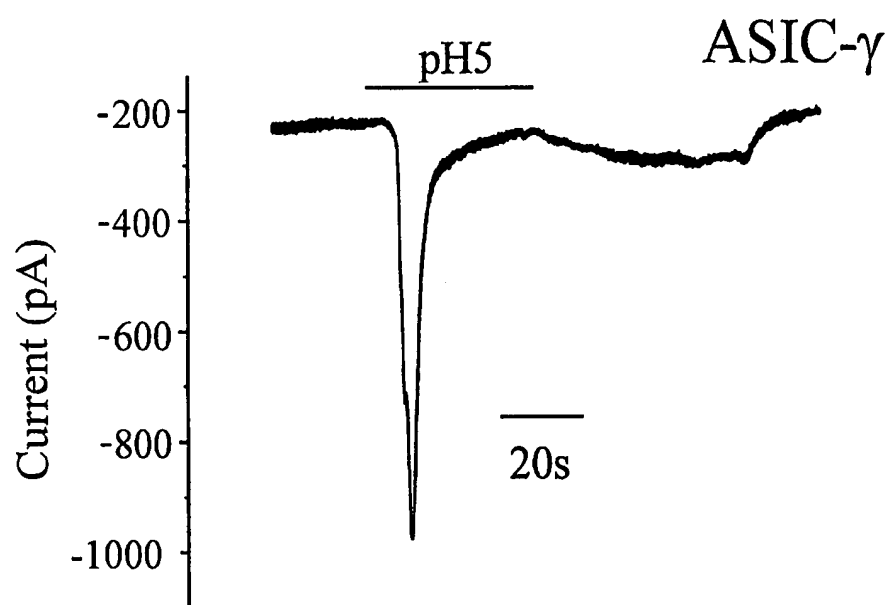


Fig.8b

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: UNIVERSITY COLLEGE LONDON

(B) STREET: GOWER STREET

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(E) COUNTRY: UNITED KINGDOM

(F) POSTAL CODE (ZIP): WC1E 6HA

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(A) NAME: CHIH CHENG CHEN

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(E) COUNTRY: UNITED KINGDOM (GB)

(F) POSTAL CODE (ZIP): WC1E 6BT

(A) NAME: ARMEN NORAKOVITCH AKOPIAN

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BIOLOGY DEPARTMENT

(C) CITY: LONDON

(E) COUNTRY: UNITED KINGDOM (GB)

(F) POSTAL CODE (ZIP): WC1E 6BT

(A) NAME: JOHN NICHOLAS WOOD

(B) STREET: UNIVERSITY COLLEGE ANATOMY/DEVELOPMENTAL
BIOLOGY DEPARTMENT

(C) CITY: LONDON

(E) COUNTRY: UNITED KINGDOM (GB)

(F) POSTAL CODE (ZIP): WC1E 6BT

(ii) TITLE OF INVENTION: ION CHANNEL PROTEINS

(iii) NUMBER OF SEQUENCES: 10

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2962 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Rattus

(F) TISSUE TYPE: Dorsal root ganglion

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 208..1746

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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CCGGGATCCA TGGCAGATAT CTGGGGTCCC CACCACCACC GGCAGCAGCA GGACAGCTCA    180

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Glu Gly Asp Asp Ser Pro Arg Asp Leu Val Ala Phe Ala Asn Ser Cys
    10                15                20

ACC CTC CAT GGT GCC AGC CAT GTG TTT GTG GAA GGG GGC CCA GGG CCA        327
Thr Leu His Gly Ala Ser His Val Phe Val Glu Gly Gly Pro Gly Pro
    25                30                35                40

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45

50

55

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Leu Cys Gln Val Gly Asp Arg Val Ala Tyr Tyr Leu Ser Tyr Pro His

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65

70

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75

80

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90

95

100

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 105 110 115 120

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125

130

135

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145

150

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160

165

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175

180

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 185 190 195 200

| | |
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| Leu Pro Val Trp Gly Glu Thr Asp Glu Thr Ser Phe Glu Ala Gly Ile | |
| 220 225 230 | |
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| Lys Val Gln Ile His Ser Gln Asp Glu Pro Pro Phe Ile Asp Gln Leu | |
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CCTGTCTCTG AATGCGTTT CCCTGTATAA AGAGTGTGGA TTCTCCCCCT GGAGTTTGTA 2846

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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 513 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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 1             5             10             15

Leu Val Ala Phe Ala Asn Ser Cys Thr Leu His Gly Ala Ser His Val
      20             25             30

Phe Val Glu Gly Gly Pro Gly Pro Arg Gln Ala Leu Trp Ala Val Ala
      35             40             45

Phe Val Ile Ala Leu Gly Ala Phe Leu Cys Gln Val Gly Asp Arg Val
      50             55             60

Ala Tyr Tyr Leu Ser Tyr Pro His Val Thr Leu Leu Asp Glu Val Ala
      65             70             75             80

Thr Ser Glu Leu Val Phe Pro Ala Val Thr Phe Cys Asn Thr Asn Ala
      85             90             95

Val Arg Leu Ser Gln Leu Ser Tyr Pro Asp Leu Leu Tyr Leu Ala Pro
      100            105            110

Met Leu Gly Leu Asp Glu Ser Asp Asp Pro Gly Val Pro Leu Ala Pro
      115            120            125

Pro Gly Pro Glu Ala Phe Ser Gly Glu Pro Phe Asn Leu His Arg Phe
      130            135            140

Tyr Asn Arg Ser Cys His Arg Leu Glu Asp Met Leu Leu Tyr Cys Ser
      145            150            155            160

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Tyr Cys Gly Gly Pro Cys Gly Pro His Asn Phe Ser Val Val Phe Thr
 165 170 175

Arg Tyr Gly Lys Cys Tyr Thr Phe Asn Ser Gly Gln Asp Gly Arg Pro
 180 185 190

Arg Leu Lys Thr Met Lys Gly Gly Thr Gly Asn Gly Leu Glu Ile Met
 195 200 205

Leu Asp Ile Gln Gln Asp Glu Tyr Leu Pro Val Trp Gly Glu Thr Asp
 210 215 220

Glu Thr Ser Phe Glu Ala Gly Ile Lys Val Gln Ile His Ser Gln Asp
 225 230 235 240

Glu Pro Pro Phe Ile Asp Gln Leu Gly Phe Gly Val Ala Pro Gly Phe
 245 250 255

Gln Thr Phe Val Ser Cys Gln Glu Gln Arg Leu Ile Tyr Leu Pro Ser
 260 265 270

Pro Trp Gly Thr Cys Asn Ala Val Thr Met Asp Ser Asp Phe Phe Asp
 275 280 285

Ser Tyr Ser Ile Thr Ala Cys Arg Ile Asp Cys Glu Thr Arg Tyr Leu
 290 295 300

Val Glu Asn Cys Asn Cys Arg Met Val His Met Pro Gly Asp Ala Pro
 305 310 315 320

Tyr Cys Thr Pro Glu Gln Tyr Lys Glu Cys Ala Asp Pro Ala Leu Asp
 325 330 335

Phe Leu Val Glu Lys Asp Gln Glu Tyr Cys Val Cys Glu Met Pro Cys
 340 345 350

Asn Leu Thr Arg Tyr Gly Lys Glu Leu Ser Met Val Lys Ile Pro Ser
 355 360 365

Lys Ala Ser Ala Lys Tyr Leu Ala Lys Lys Phe Asn Lys Ser Glu Gln
 370 375 380

Tyr Ile Gly Glu Asn Ile Leu Val Leu Asp Ile Phe Phe Glu Val Leu
 385 390 395 400

Asn Tyr Glu Thr Ile Glu Gln Lys Lys Ala Tyr Glu Ile Ala Gly Leu
 405 410 415

Leu Gly Asp Ile Gly Gly Gln Met Gly Leu Phe Ile Gly Ala Ser Ile
 420 425 430

Leu Thr Val Leu Glu Leu Phe Asp Tyr Ala Tyr Glu Val Ile Lys His
 435 440 445

Arg Leu Cys Arg Arg Gly Lys Cys Gln Lys Glu Ala Lys Arg Ser Ser
 450 455 460

Ala Asp Lys Gly Val Ala Leu Ser Leu Asp Asp Val Lys Arg His Asn
 465 470 475 480

Pro Cys Glu Ser Leu Arg Gly His Pro Ala Gly Met Thr Tyr Ala Ala
 485 490 495

Asn Ile Leu Pro His His Pro Ala Arg Gly Thr Phe Glu Asp Phe Thr
 500 505 510

Cys

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 960 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Rattus

(F) TISSUE TYPE: Dorsal root ganglion

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:1..960

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ATG GAA TTG AAG ACC GAG GAG GAG GAG GTG GGT GGT GTC CAG CCG GTG 48
Met Glu Leu Lys Thr Glu Glu Glu Glu Val Gly Gly Val Gln Pro Val
515 520 525

AGC ATC CAG GCT TTC GCC AGC AGC TCC ACG CTG CAT GGT CTT GCC CAC 96
Ser Ile Gln Ala Phe Ala Ser Ser Ser Thr Leu His Gly Leu Ala His
530 535 540 545

ATC TTC TCC TAT GAG CGG CTG TCT CTG AAG CGG GCA CTG TGG GCC CTG 144
Ile Phe Ser Tyr Glu Arg Leu Ser Leu Lys Arg Ala Leu Trp Ala Leu
550 555 560

TGC TTC CTG GGT TCG CTG GCC GTC CTG CTG TGT GTG TGC ACT GAG CGT 192
Cys Phe Leu Gly Ser Leu Ala Val Leu Leu Cys Val Cys Thr Glu Arg
565 570 575

GTG CAG TAC TAC TTC TGC TAT CAC CAC GTC ACC AAG CTT GAC GAA GTG 240
Val Gln Tyr Tyr Phe Cys Tyr His His Val Thr Lys Leu Asp Glu Val
580 585 590

GCT GCC TCC CAG CTC ACC TTC CCT GCT GTC ACA CTG TGC AAT CTC AAT 288
Ala Ala Ser Gln Leu Thr Phe Pro Ala Val Thr Leu Cys Asn Leu Asn
595 600 605

GAG TTC CGC TTT AGC CAA GTC TCC AAG AAT GAC CTG TAC CAT GCT GGG 336
Glu Phe Arg Phe Ser Gln Val Ser Lys Asn Asp Leu Tyr His Ala Gly
610 615 620 625

GAG CTG CTG GCC CTG CTC AAC AAC AGG TAT GAG ATC CCG GAC ACA CAG 384
 Glu Leu Leu Ala Leu Leu Asn Asn Arg Tyr Glu Ile Pro Asp Thr Gln
 630 635 640

ATG GCT GAT GAA AAG CAG CTA GAG ATA TTG CAG GAC AAG GCC AAC TTC 432
 Met Ala Asp Glu Lys Gln Leu Glu Ile Leu Gln Asp Lys Ala Asn Phe
 645 650 655

CGG AGC TTC AAG CCC AAG CCC TTC AAC ATG CGT GAA TTC TAC GAC AGA 480
 Arg Ser Phe Lys Pro Lys Pro Phe Asn Met Arg Glu Phe Tyr Asp Arg
 660 665 670

GCG GGG CAC GAT ATT CGA GAC ATG CTG CTC TCG TGC CAC TTC CGT GGG 528
 Ala Gly His Asp Ile Arg Asp Met Leu Leu Ser Cys His Phe Arg Gly
 675 680 685

GAG GCC TGC AGC GCT GAA GAT TTC AAA GTG GTC TTC ACT CGG TAT GGG 576
 Glu Ala Cys Ser Ala Glu Asp Phe Lys Val Val Phe Thr Arg Tyr Gly
 690 695 700 705

AAG TGT TAC ACA TTC AAC TCG GGC CAA GAT GGG CGG CCA CGG CTG AAG 624
 Lys Cys Tyr Thr Phe Asn Ser Gly Gln Asp Gly Arg Pro Arg Leu Lys
 710 715 720

ACC ATG AAA GGT GGG ACT GGC AAT GGC CTG GAG ATC ATG CTG GAC ATT 672
 Thr Met Lys Gly Gly Thr Gly Asn Gly Leu Glu Ile Met Leu Asp Ile
 725 730 735

CAG CAA GAT GAA TAT TTG CCT GTG TGG GGA GAG ACC GGT ATG TCC GGT 720
 Gln Gln Asp Glu Tyr Leu Pro Val Trp Gly Glu Thr Gly Met Ser Gly
 740 745 750

CTG CTT AAG GAA CAC CTT CCT TGG GCC CGC CTG GCC CCA AAC TCA ATT 768
 Leu Leu Lys Glu His Leu Pro Trp Ala Arg Leu Ala Pro Asn Ser Ile
 755 760 765

AGG GCT CCA CTG TGC AGC CCT GGC ACC AAA TTC AGA CAC GTC CCC TTT 816
 Arg Ala Pro Leu Cys Ser Pro Gly Thr Lys Phe Arg His Val Pro Phe
 770 775 780 785

CTC CCA AGA TCT CAC CAT GTC TGT CTT TGC CCC TAT GTG ACT TCC GCA 864
 Leu Pro Arg Ser His His Val Cys Leu Cys Pro Tyr Val Thr Ser Ala
 790 795 800

CTC TCG GTT CCC TGG CTC CTC CTT CCT GGT TTA GTT ACT CTC CAA GGT 912
 Leu Ser Val Pro Trp Leu Leu Leu Pro Gly Leu Val Thr Leu Gln Gly
 805 810 815

AAC CAG GCA TCC TCT TCC ATC AGT GGC CGC CAC TCA CTC TAT AAA TAA 960
 Asn Gln Ala Ser Ser Ser Ile Ser Gly Arg His Ser Leu Tyr Lys *
 820 825 830

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 320 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Met Glu Leu Lys Thr Glu Glu Glu Glu Val Gly Gly Val Gln Pro Val
 1 5 10 15

Ser Ile Gln Ala Phe Ala Ser Ser Ser Thr Leu His Gly Leu Ala His
 20 25 30

Ile Phe Ser Tyr Glu Arg Leu Ser Leu Lys Arg Ala Leu Trp Ala Leu
 35 40 45

Cys Phe Leu Gly Ser Leu Ala Val Leu Leu Cys Val Cys Thr Glu Arg
 50 55 60

Val Gln Tyr Tyr Phe Cys Tyr His His Val Thr Lys Leu Asp Glu Val
 65 70 75 80

Ala Ala Ser Gln Leu Thr Phe Pro Ala Val Thr Leu Cys Asn Leu Asn
 85 90 95

Glu Phe Arg Phe Ser Gln Val Ser Lys Asn Asp Leu Tyr His Ala Gly
 100 105 110

Glu Leu Leu Ala Leu Leu Asn Asn Arg Tyr Glu Ile Pro Asp Thr Gln
 115 120 125

Met Ala Asp Glu Lys Gln Leu Glu Ile Leu Gln Asp Lys Ala Asn Phe
 130 135 140

Arg Ser Phe Lys Pro Lys Pro Phe Asn Met Arg Glu Phe Tyr Asp Arg
 145 150 155 160

Ala Gly His Asp Ile Arg Asp Met Leu Leu Ser Cys His Phe Arg Gly
 165 170 175

Glu Ala Cys Ser Ala Glu Asp Phe Lys Val Val Phe Thr Arg Tyr Gly
 180 185 190

Lys Cys Tyr Thr Phe Asn Ser Gly Gln Asp Gly Arg Pro Arg Leu Lys
 195 200 205

Thr Met Lys Gly Gly Thr Gly Asn Gly Leu Glu Ile Met Leu Asp Ile
 210 215 220

Gln Gln Asp Glu Tyr Leu Pro Val Trp Gly Glu Thr Gly Met Ser Gly
 225 230 235 240

Leu Leu Lys Glu His Leu Pro Trp Ala Arg Leu Ala Pro Asn Ser Ile
 245 250 255

Arg Ala Pro Leu Cys Ser Pro Gly Thr Lys Phe Arg His Val Pro Phe
 260 265 270

Leu Pro Arg Ser His His Val Cys Leu Cys Pro Tyr Val Thr Ser Ala
 275 280 285

Leu Ser Val Pro Trp Leu Leu Leu Pro Gly Leu Val Thr Leu Gln Gly
 290 295 300

Asn Gln Ala Ser Ser Ser Ile Ser Gly Arg His Ser Leu Tyr Lys *
 305 310 315 320

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 526 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Rattus
- (F) TISSUE TYPE: Dorsal root ganglion

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Met Glu Leu Lys Thr Glu Glu Glu Glu Val Gly Gly Val Gln Pro Val
 1 5 10 15

Ser Ile Gln Ala Phe Ala Ser Ser Ser Thr Leu His Gly Leu Ala His
 20 25 30

Ile Phe Ser Tyr Glu Arg Leu Ser Leu Lys Arg Ala Leu Trp Ala Leu
 35 40 45

Cys Phe Leu Gly Ser Leu Ala Val Leu Leu Cys Val Cys Thr Glu Arg
 50 55 60

Val Gln Tyr Tyr Phe Cys Tyr His His Val Thr Lys Leu Asp Glu Val
 65 70 75 80

Ala Ala Ser Gln Leu Thr Phe Pro Ala Val Thr Leu Cys Asn Leu Asn
 85 90 95

Glu Phe Arg Phe Ser Gln Val Ser Lys Asn Asp Leu Tyr His Ala Gly
 100 105 110

Glu Leu Leu Ala Leu Leu Asn Asn Arg Tyr Glu Ile Pro Asp Thr Gln
 115 120 125

Met Ala Asp Glu Lys Gln Leu Glu Ile Leu Gln Asp Lys Ala Asn Phe
 130 135 140

Arg Ser Phe Lys Pro Lys Pro Phe Asn Met Arg Glu Phe Tyr Asp Arg
 145 150 155 160

Ala Gly His Asp Ile Arg Asp Met Leu Leu Ser Cys His Phe Arg Gly
 165 170 175

Glu Ala Cys Ser Ala Glu Asp Phe Lys Val Val Phe Thr Arg Tyr Gly
 180 185 190

Lys Cys Tyr Thr Phe Asn Ser Gly Gln Asp Gly Arg Pro Arg Leu Lys
 195 200 205

Thr Met Lys Gly Gly Thr Gly Asn Gly Leu Glu Ile Met Leu Asp Ile
 210 215 220

Gln Gln Asp Glu Tyr Leu Pro Val Trp Gly Glu Thr Asp Glu Thr Ser
 225 230 235 240

Phe Glu Ala Gly Ile Lys Val Gln Ile His Ser Gln Asp Glu Pro Pro
 245 250 255

Phe Ile Asp Gln Leu Gly Phe Gly Val Ala Pro Gly Phe Gln Thr Phe
 260 265 270

Val Ser Cys Gln Glu Gln Arg Leu Ile Tyr Leu Pro Ser Pro Trp Gly
 275 280 285

Thr Cys Asn Ala Val Thr Met Asp Ser Asp Phe Phe Asp Ser Tyr Ser
 290 295 300

Ile Thr Ala Cys Arg Ile Asp Cys Glu Thr Arg Tyr Leu Val Glu Asn

| | | | |
|---|-----|-----|-----|
| 305 | 310 | 315 | 320 |
| Cys Asn Cys Arg Met Val His Met Pro Gly Asp Ala Pro Tyr Cys Thr | | | |
| 325 | 330 | 335 | |

| | | |
|---|-----|-----|
| Pro Glu Gln Tyr Lys Glu Cys Ala Asp Pro Ala Leu Asp Phe Leu Val | | |
| 340 | 345 | 350 |

| | | |
|---|-----|-----|
| Glu Lys Asp Gln Glu Tyr Cys Val Cys Glu Met Pro Cys Asn Leu Thr | | |
| 355 | 360 | 365 |

| | | |
|---|-----|-----|
| Arg Tyr Gly Lys Glu Leu Ser Met Val Lys Ile Pro Ser Lys Ala Ser | | |
| 370 | 375 | 380 |

| | | |
|---|-----|-----|
| Ala Lys Tyr Leu Ala Lys Lys Phe Asn Lys Ser Glu Gln Tyr Ile Gly | | |
| 385 | 390 | 395 |
| | | 400 |

| | | |
|---|-----|-----|
| Glu Asn Ile Leu Val Leu Asp Ile Phe Phe Glu Val Leu Asn Tyr Glu | | |
| 405 | 410 | 415 |

| | | |
|---|-----|-----|
| Thr Ile Glu Gln Lys Lys Ala Tyr Glu Ile Ala Gly Leu Leu Gly Asp | | |
| 420 | 425 | 430 |

| | | |
|---|-----|-----|
| Ile Gly Gly Gln Met Gly Leu Phe Ile Gly Ala Ser Ile Leu Thr Val | | |
| 435 | 440 | 445 |

| | | |
|---|-----|-----|
| Leu Glu Leu Phe Asp Tyr Ala Tyr Glu Val Ile Lys His Arg Leu Cys | | |
| 450 | 455 | 460 |

| | | |
|---|-----|-----|
| Arg Arg Gly Lys Cys Gln Lys Glu Ala Lys Arg Ser Ser Ala Asp Lys | | |
| 465 | 470 | 475 |
| | | 480 |

| | | |
|---|-----|-----|
| Gly Val Ala Leu Ser Leu Asp Asp Val Lys Arg His Asn Pro Cys Glu | | |
| 485 | 490 | 495 |

| | | |
|---|-----|-----|
| Ser Leu Arg Gly His Pro Ala Gly Met Thr Tyr Ala Ala Asn Ile Leu | | |
| 500 | 505 | 510 |

| | | |
|---|-----|-----|
| Pro His His Pro Ala Arg Gly Thr Phe Glu Asp Phe Thr Cys | | |
| 515 | 520 | 525 |

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Rattus

(F) TISSUE TYPE: Dorsal root ganglion

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GGGGAGCCTT TTAACCTCCA TCGTTTCT

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(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Rattus

(F) TISSUE TYPE: Dorsal root ganglion

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

ACCATACGGC AGTTGCAGTT CTC

23

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (iii) HYPOTHETICAL: NO
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Rattus
 - (F) TISSUE TYPE: Dorsal root ganglion
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TGTACTGCTC TGGAGTGCAG TAT

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(2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Rattus
 - (F) TISSUE TYPE: Dorsal root ganglion

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

ACTGGCAATG GCCTGGAGAT CATG

24

(2) INFORMATION FOR SEQ ID NO: 10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Rattus
 - (F) TISSUE TYPE: Dorsal root ganglion
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GGTCCAAGCA CACCTTCAGC CTC

23

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 98/02609

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C07K14/705 C07K16/28 A61K38/17 A61K48/00
G01N33/68 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|---|---------------------------|
| X | WALDMANN R ET AL: "THE MAMMALIAN DEGENERIN MDEG, AN AMILORIDE-SENSITIVE CATION CHANNEL ACTIVATED BY MUTATIONS CAUSING NEURODEGENERATION IN CAENORHABDITIS ELEGANS" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 18, 3 May 1996, pages 10433-10436, XP002051361 cited in the application | 16,17 |
| A | see the whole document

-/-- | 1,5,7,
14,18,
21,23 |

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search

2 December 1998

Date of mailing of the international search report

21/12/1998

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Authorized officer

Gurdjian, D

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 98/02609

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|--|---------------------------|
| X | PRICE MP ET AL: "Cloning and expression of a novel human brain Na ⁺ channel."
J BIOL CHEM, APR 5 1996, 271 (14)
P7879-82, XP002068541
UNITED STATES | 16,17 |
| A | see the whole document | 1,5,7,
14,18,
21,23 |
| X | WALDMANN R ET AL: "A proton-gated cation channel involved in acid-sensing."
NATURE, MAR 13 1997, 386 (6621) P173-7;
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ENGLAND | 16,17 |
| A | cited in the application
see the whole document | 1,5,7,
14,18,
21,23 |
| X | GARCIA-ANOVEROS J ET AL: "BNAC1 AND BNAC2 CONSTITUTE A NEW FAMILY OF HUMAN NEURONAL SODIUM CHANNELS RELATED TO DEGENERINS AND EPITHELIAL SODIUM CHANNELS"
PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA,
vol. 94, no. 4, 18 February 1997, pages 1459-1464, XP002051359 | 16,17 |
| A | cited in the application
see the whole document | 1,5,7,
14,18,
21,23 |
| P,X | WO 98 35034 A (HEURTEAUX CATHERINE
;CHAMPIGNY GUY (FR); LINGUEGLIA ERIC (FR);
WAL) 13 August 1998
see abstract; claims 1-25; figures SEQ.,4 | 1-29,
34-42 |
| P,X | CHEN CC ET AL: "A sensory neuron-specific, proton-gated ion channel."
PROC NATL ACAD SCI U S A, AUG 18 1998, 95 (17) P10240-5, XP002086506
UNITED STATES | 1-29 |
| | see the whole document | |

Information on patent family members

PCT/GB 98/02609

Form PCT/ISA/210 (patent family annex) (July 1992)